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July 29, 2004	Shelley P.M. Fussey
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Philip E. Thorpe, Sophia Ran and Rolf A. Brekken (As Amended)

Group Art Unit: 1619

Serial No.: 09/351,149

Examiner: Sharareh, S.

Filed: July 12, 1999

Atty. Dkt. No.: 3999.002383

For: CANCER TREATMENT KITS COMPRISING THERAPEUTIC CONJUGATES THAT BIND TO AMINOPHOSPHOLIPIDS

BRIEF ON APPEAL

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BRIEF ON APPEAL

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the Fifth and Final Official Action dated July 15, 2003 ("the fifth Action"). The Notice of Appeal was submitted on December 30, 2003, and was received in the Office on January 02, 2004. The two month date for filing the Appeal Brief was March 02, 2004 and the Brief is timely filed within the extendable period thereafter. The large entity fee for filing this Appeal Brief is $330, which is enclosed herewith.

If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. § 1.16 to 37 C.F.R. §1.21 be required for any reason relating to the enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Williams, Morgan & Amerson, P.C. Deposit Account No. 50-0786/3999.002383.

I. REAL PARTY IN INTEREST

The real parties in interest are the Assignee of the application, Board of Regents, The University of Texas System, and the exclusive licensee, Peregrine Pharmaceuticals, Inc.

II. RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any other appeals or interferences related to the present application.

III. STATUS OF THE CLAIMS

Claims 1-43 were filed with the original application. In a first Restriction Requirement dated January 19, 2000, the original claims were said to be drawn to three distinct inventions. In a response submitted January 31, 2000, Appellants suggested clarification of the Restriction Requirement and provisionally elected the Group I invention, then said to contain claims 1-19, 42 and 43.

In a second Restriction and Species Election Requirement dated May 09, 2000, the Office separated claims 1-43 into three different groups. In a response submitted August 03, 2000, Appellants elected the Group I invention without traverse, which then contained claims 1-32 and 43. Claims 33-42 were canceled as drawn to non-elected inventions and claim 44 was added. In reply to the requirement to elect particular species for initial examination, Appellants made a number of species elections without traverse. At the end of this phase, claims 1-32, 43 and 44 were pending; claims 1-9, 16-19, 24-32 and 43 read on the elected species; and claims 10-15, 20-23 and 44 remained pending and available for rejoinder upon the allowance of a generic claim, as provided by 37 C.F.R. § 1.141.

The first Official Action, dated November 07, 2000, examined claims 1-9, 16-19, 24-32 and 43 on the merits. The first Action rejected all examined claims under 35 U.S.C. § 112, second paragraph; under the judicially created doctrine of obviousness-type double patenting; and under over three different combinations of references (Huang and Martin; Gimbrone and Dvorak; and Gimbrone and Huang). On December 06, 2000, Appellants responded to the rejections, amended claim 43 and added claims 45-48, which were entered and examined on the merits.

In a second and non-final Official Action dated March 13, 2001, claims 1-9, 16-19, 24-32, 43 and 45-48 were examined. All rejections from the first Action were withdrawn. All examined claims were subject to a new rejection under 35 U.S.C. § 112, second paragraph; to a new provisional obviousness-type double patenting rejection over co-pending application Serial No. 09/351,457; and to a new rejection under 35 U.S.C. § 103(a) over Blankenberg, Huang, WO 98/29453 and Fishman. On September 13, 2001, Appellants responded to the rejections,

submitted a declaration under 37 C.F.R. § 1.131 to remove WO 98/29453 as prior art, and added claim 49, which was entered and examined on the merits.

The third and non-final Official Action, dated December 31, 2001, examined claims 1-9, 14, 16-19, 24-32, 43 and 45-49. The provisional obviousness-type double patenting rejection and the § 103(a) rejection were withdrawn. Claim 32 was said to be free of the art. The § 112, second paragraph rejection of claims 1-9, 16-19, 24-32, 43 and 45-48 was maintained; claims 1, 3-5 and 8 were newly rejected under 35 U.S.C. § 102(b) over Fishman; claims 1-9 and 14 were newly rejected under 35 U.S.C. § 102(e) over Schroit; claims 1-9, 16-19, 24-31, 43 and 45-49 were newly rejected under 35 U.S.C. § 103(a) over Schroit, Gimbrone, Blankenberg and Umeda; and all examined claims were subject to a new provisional obviousness-type double patenting rejection over co-pending application Serial No. 09/351,862 ("the '862 application"). On June 12, 2002, Appellants responded to the rejections, amended claims 1, 32 and 43, and added claim 50, which was entered and examined on the merits.

In a fourth and non-final Official Action dated October 22, 2002, claims 1-9, 14, 16-19, 24-32, 43 and 45-50 were examined. The § 112, second paragraph rejection, the § 102(b) rejection over Fishman and the § 103(a) rejection over Schroit, Gimbrone, Blankenberg and Umeda were withdrawn. Claim 32 was said to be free of the art. The rejection of claims 1-9 and 14 under 35 U.S.C. § 102(e) over Schroit was maintained; claims 1-9, 16-19, 24-31, 43 and 45-50 were newly rejected under 35 U.S.C. § 103(a) over Schroit, Gimbrone and Blankenberg; and the provisional obviousness-type double patenting rejection of claims 1-9, 16-19, 24-32, 43 and 45-49 over the '862 application was maintained. On April 22, 2003, Appellants responded to the rejections and added claims 51-57, which were entered and examined on the merits.

On July 15, 2003, a fifth Official Action was mailed and made final. Claims 1-9, 16-19, 24-32, 43 and 45-57 were examined. The § 102(e) rejection over Schroit and the § 103(a)rejection over Schroit, Gimbrone and Blankenberg were withdrawn. Claim 43 was allowed and claim 32 was only objected to (although both were included in the provisional double patenting rejection). Claims 1-9, 16-19, 24-31 and 45-57 were newly rejected under 35 U.S.C. § 103(a) over Schroit, Gimbrone, Blankenberg and Abrams. The provisional obviousness-type double patenting rejection of claims 1-9, 16-19, 24-32, 43 and 45-49 over the '862 application was maintained.

The fifth Action was prematurely final as it entered a new ground of rejection using the Abrams patent, properly and timely submitted in an Information Disclosure Statement under 37 C.F.R. § 1.97(c) without a fee. On August 15, 2003, Appellants submitted a request to withdraw the holding of finality. Appellants' request was denied in an Advisory Action mailed October 31, 2003. On December 30, 2003, Appellants submitted a Notice of Appeal.

Accordingly, claims 1-32 and 43-57 are pending. The Office holds claims 10-15, 20-23 and 44 to be withdrawn from consideration. Claims 1-9, 16-19, 24-32, 43 and 45-57 are undergoing active examination, of which claim 43 is allowed and claim 32 is objected to. Claims 1-9, 16-19, 24-31 and 45-57 are rejected and appealed. Claims 43 and 32, despite being allowed and allowable, respectively, are provisionally rejected for obviousness-type double patenting and are included with the claims on appeal.

IV. STATUS OF AMENDMENTS

Subsequent to the final rejection, Appellants considered submitting an amendment to cancel or revise the diagnostic kit claims, which the Office holds to be directed to non-elected species and thus withdrawn (and to correct a clerical oversight in claim 55). A draft of such an

amendment was forwarded via facsimile for the examiner's consideration, but Appellants did not seek entry of the draft amendment.

Therefore, no amendment has been filed subsequent to the final rejection.

Claims 1-32 and 43-57 are pending; claims 10-15, 20-23 and 44 are withdrawn from consideration; claim 43 is allowed; and claim 32 is objected to. Claims 1-9, 16-19, 24-31 and 45-57 are rejected and appealed; claims 43 and 32, despite being allowed and allowable, are provisionally rejected and included in the appeal. The claims on appeal are set forth in **Appendix A**. For the Board's convenience, pending claims 1-32 and 43-57 are set forth in **Appendix B**, which includes the status of the claims that are allowed, objected to and withdrawn.

V. SUMMARY OF THE INVENTION

The present application is one of a family of four U.S. patents and applications, which generally relate to the fields of blood vessels and tumor biology (specification at page 2, line 10). Solid tumors contain a network of blood vessels, or vasculature, which provides oxygen and nutrients to the tumor cells. Therapeutic agents that attack or destroy the tumor vasculature have been used effectively in cancer treatment, expanding the treatment options from the classic "chemotherapeutic agents", which act on the tumor cells themselves (specification at pages 2-4). To deliver therapeutic agents specifically to the tumor vasculature, markers are needed that are expressed preferentially on tumor blood vessels as opposed to blood vessels in normal tissues (specification at pages 3-4).

The invention of the present and related patents and applications is based, in part, on the inventors' surprising findings that biological components termed "aminophospholipids", such as phosphatidylserine and phosphatidylethanolamine, are accessible, stable and specific markers of blood vessels within tumors, as opposed to normal vasculature (specification throughout,

including Examples VIII, IX, X, XI, XII, XIII and XIV). The inventors developed new therapeutic constructs, and associated methods and kits, to specifically deliver therapeutic agents to vascularized tumors by virtue of binding to these newly discovered markers of the tumor vasculature (specification throughout, *e.g.*, at pages 4-5).

These new therapeutic constructs comprise a targeting agent that binds to the aminophospholipid, operatively attached to a therapeutic agent. Such therapeutic constructs or "binding ligands" localize to tumor vasculature by binding to the aminophospholipids on the tumor blood vessels, and deliver the attached therapeutic agent, such as a toxin or coagulant, into close contact with the tumor blood vessels, where it exerts its therapeutic effect (specification at pages 4-5). The therapeutic constructs may be used in range of embodiments, particularly in tumor treatment, both alone and in combination with other anti-cancer agents (specification at pages 4-44).

To seek protection of the overall invention, three U.S. patent applications were filed on the same day, each having the same specification. The claims of these three applications were separately drawn to the compositions, methods and kits of the invention (in a manner that was believed to be consistent with U.S. restriction practice). A continuation with additional method claims was filed upon allowance of the first method claim application.

The composition claims for the new therapeutic constructs or binding ligands were submitted in application Serial No. 09/351,598 ("the '598 application"), having the same specification and filing date as the present case. The composition claims, which are directed to binding ligands that comprise at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent, were allowed in 2002 (Notice of

Allowance mailed June 04, 2002; issue fee paid June 11, 2002)[1]. The allowed composition claims of the '598 application, which represent the main component of the kits claimed in the present application and subject to this appeal, are enclosed as **Exhibit A**.

Claims directed to methods of using the new therapeutic agents were submitted in application Serial No. 09/351,457 ("the '457 application"), also having the same specification and filing date as the present case. The method claims in the '457 application, which include delivering selected therapeutic agents to tumor vasculature and treating cancer by administering a binding ligand that comprises at least a first therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid, were allowed and issued as U.S. Patent No. 6,312,694 ("the '694 patent") on November 06, 2001.

The method claims issued from the '457 application are enclosed as **Exhibit B**. During examination of the present application, a provisional obviousness-type double patenting rejection of all claims was entered over the claims in the '457 application. The provisional double patenting rejection was withdrawn when Appellants pointed out that the claims in the '457 application had been allowed[2].

Further method claims were filed in application Serial No. 09/819,386 ("the '386 application"), a continuation of the '457 application, which have also been allowed recently (issue fee paid March 10, 2004). These claims, which are directed to combination methods for treating cancer, include simultaneously or sequentially administering a targeting agent-therapeutic agent construct of the overall invention, *i.e.*, one that binds to an aminophospholipid, and at least a second anti-cancer agent. The allowed method claims in the '386 application,

[1]The file wrapper for the '598 application has been lost twice by the Office, re-submitted twice by Appellants and the application has yet to issue.
[2]Typically, a provisional double patenting rejection would be maintained on allowance of the cited application and converted from a provisional rejection to an actual double patenting rejection when the allowed application issues.

which are enclosed as **Exhibit C**, represent *in vivo* methods of using the kits claimed in the present application and subject to this appeal.

The present claims are directed to kits comprising an aminophospholipid targeting agent-therapeutic agent construct, additionally defined in the claims as a "first anti-cancer agent", in combination with a second anti-cancer agent or a detectable agent construct that binds to an aminophospholipid (*e.g.*, claims 1 and 49). The "diagnostic kits", drawn solely to combinations with detectable agent constructs (claims 20-23 and 44), have been withdrawn from consideration as drawn to non-elected species and are not the focus of this appeal. Kits that comprise the aminophospholipid targeting agent-therapeutic agent construct; a second anti-cancer agent; and an aminophospholipid targeting agent-detectable agent construct are allowed or allowable (claims 43 and 32, respectively).

The "therapeutic kits" of the appeal comprise a binding ligand or "aminophospholipid targeting agent-therapeutic agent construct", defined as the first anti-cancer agent, in combination with a second anti-cancer agent "other than" the aminophospholipid targeting agent-therapeutic agent construct (*e.g.*, claim 1(b), claim 49(b) and claim 51). Certain kits recite combinations with particular second anti-cancer agents, currently preferred for combined use with the aminophospholipid targeting agent constructs (*e.g.*, claims 53-57; see also specification from page 39, line 15 to page 40, line 11; see also Section J, from pages 151-173).

As in the three related cases, the targeting agent-therapeutic agent constructs of the claimed kits comprise "at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent" (present claims 1, 43, 49, 51 and 53). This definition matches the allowed and issued claims in the related applications (*e.g.*, claims 1,

39, 53 and 59 in the '598 application; claims 4, 47 and 50 in the '694 patent; and claims 5 and 65 in the '386 application).

The targeting agent portion of the targeting agent-therapeutic agent construct may comprise a targeting agent that binds to phosphatidylethanolamine (claims 2, 12 and 52) or to phosphatidylserine (claims 3 and 11). Counterpart claims were allowed or issued in the related applications (*e.g.*, at least claims 2, 3, 19, 20, 21 and 22, in the '598 application; at least claims 5, 6, 21, 22, 23 and 24 in the '694 patent; and claims 6, 7, 81 and 82 in the '386 application).

Antibodies, or antigen-binding fragments thereof, are suitable for use as the targeting agent portion of the targeting agent-therapeutic agent, as in claims 4-9. Alternatively, aminophospholipid binding proteins may be used as the targeting agent, as in claims 10-13. Claims to targeting agent-therapeutic agent constructs with both antibodies and binding proteins were allowed or issued in the related cases (*e.g.*, at least claims 4-17, 18-22, 40, 49 and 52 in the '598 application; at least claims 7-19 and 20-24 in the '694 patent; and claims 8-12, 21 and 81-83 in the '386 application).

Suitable therapeutic agents for use in the targeting agent-therapeutic agent construct include anticellular or cytotoxic agents (claims 14 and 15) and coagulants (claims 16-18). Claims to targeting agent-therapeutic agent constructs with a range of cytotoxic agents and coagulants have been allowed or issued in the related applications (*e.g.*, at least claims 24-28 and 29-33 in the '598 application; at least claims 28-32 and 33-37 in the '694 patent; and claims 29-30 and 34-38 in the '386 application).

Exemplary second anti-cancer agents for use in the claimed kits include chemotherapeutic, radiotherapeutic, anti-angiogenic and apoptosis-inducing agents (claim 27) and immunoconjugates that bind to tumor cells, stroma or vasculature (claims 28-31). Such

second anti-cancer agents are also included in the claims allowed or issued in the related cases (*e.g.*, composition claims 57, 61, 62 and 63 in the '598 application; and method claims 5, 47-57, 65-80 and 84-90 in the '386 application).

Claims 53-57 of the present application are directed to kits of the aminophospholipid targeting agent-therapeutic agent constructs in combination with particular second anti-cancer agents, currently preferred for simultaneous or sequential administration in tumor treatment. These second anti-cancer agents increase aminophospholipid expression in the tumor blood vessel; injure or induce apoptosis in the tumor blood vessels; kill tumor cells; or are anti-angiogenic agents that inhibit metastasis of tumor cells. The method claims allowed in the '386 application, which are the *in vivo* method counterparts to the present kit claims, include claims to the same preferred second anti-cancer agents (*e.g.*, '386 application at claims 48, 69, 70, 72-75, 77-80 and 84-90).

The claims as a whole therefore define kits comprising binding ligands or aminophospholipid targeting agent-therapeutic agent constructs in combination with other (second) anti-cancer agents. Compositions comprising the aminophospholipid targeting agent-therapeutic agent constructs, optionally in combination with second anti-cancer agents, have been allowed in the related, '598 application (**Exhibit A**). Methods of using the aminophospholipid targeting agent-therapeutic agent constructs to treat cancer have issued as the '694 patent (**Exhibit B**), and combination treatment methods using the aminophospholipid targeting agent-therapeutic agent constructs and second anti-cancer agents have been allowed in the related, '386 application (**Exhibit C**).

VI. ISSUES ON APPEAL

A. Whether claims 1-9, 16-19, 24-31 and 45-57 are legally obvious under 35 U.S.C. § 103(a) over U.S. Patent No. 6,300,308 to Schroit ("Schroit") in view of U.S. Patent No. 5,632,991 to Gimbrone ("Gimbrone"), U.S. Patent No. 6,197,278 to Blankenberg *et al.* ("Blankenberg"), and U.S. Patent No. 4,867,962 to Abrams ("Abrams"); and

B. Whether a proper provisional rejection under the judicially created doctrine of obviousness-type double patenting35 U.S.C. § 103(a) has been established for claims 1-9, 16-19, 24-32, 43 and 45-49 over "pending claim" of co-pending Application Serial No. 09/351,862 ("the '862 application").

VII. GROUPING OF THE CLAIMS

Certain claims stand or fall separately according to the issues on appeal. Appellants group the claims as follows, for the stated reasons:

A. For the rejection under 35 U.S.C. § 103(a) over Schroit, Gimbrone, Blankenberg and Abrams, claims 1, 3-9, 19, 24-26, 50 and 51 stand together as the rejection is overcome by the features in common to these claims; claims 2 and 52; claims 16-18; claim 27; claims 28-31; claims 45-48; claim 49; and claims 53-57 stand separately, each from the others, as they recite particular features that are not taught or suggested by the cited art; and

B. For the provisional obviousness-type double patenting rejection, claims 1-9, 16-19, 24-32, 43 and 45-49 stand together as the rejection, if proper, will be overcome by filing a Terminal Disclaimer directed to all claims.

For the sake of clarity, Appellants' grouping of the claims in the foregoing manner is brought out in the following arguments specifically addressing each rejection.

VIII. ARGUMENT

A. Claims 1-9, 16-19, 24-31 and 45-57 are Not Obvious Under 35 U.S.C. § 103(a) Over Schroit, Gimbrone, Blankenberg and Abrams

The fifth Action newly rejects claims 1-9, 16-19, 24-31 and 45-57 under 35 U.S.C. § 103(a) as allegedly being legally obvious over Schroit in view of Gimbrone, Blankenberg and Abrams.

Claim 43 is allowed (fifth Action at summary page) or at least free from the art (fifth Action at page 2). As claim 43 is allowed or free from the art, each of claims 45-48, which

depend on claim 43, must also be allowed or free from the art. The rejection of the remaining claims is also unfounded and should be reversed, as set forth below[3].

The presently claimed invention is directed to kits comprising a first anti-cancer agent in the form of an aminophospholipid targeting agent-therapeutic agent construct and a second anti-cancer agent other than such a targeting agent-therapeutic agent construct (*e.g.*, claims 1 and 51), *i.e.*, a second, distinct anti-cancer agent. The targeting agent-therapeutic agent constructs comprise a targeting agent that binds to an aminophospholipid, such as phosphatidylserine (PS) or phosphatidylethanolamine (PE), surprisingly found to be accessible, stable and specific markers on the luminal surface of tumor blood vessels (claim 49; specification at pages 4-5; Examples VIII through XIV). Certain kits recite particular second anti-cancer agents, currently preferred for simultaneous or sequential administration (*e.g.*, claims 53-57; specification at pages 39-40 and pages 151-173). The kits may optionally further comprise a targeting agent-detectable agent construct that binds to an aminophospholipid (allowed and allowable claims 43 and 32).

The four references cited against the claimed invention under 35 U.S.C. § 103(a) have been improperly combined. Even if properly combined, the references in combination fail to teach or suggest or the claimed invention, fail to provide a reasonable expectation of success and, in many regards, teach away from the invention. The § 103(a) rejection is therefore improper and should be reversed.

1. Withdrawal of Rejections Under 35 U.S.C. § 102(e) and § 103(a)

The fourth Action rejected claims 1-9 and 14 under 35 U.S.C. § 102(e) as allegedly anticipated by Schroit; and rejected claims 1-9, 16-19, 24-31, 43 and 45-50 under 35 U.S.C.

[3]Appellants have noted that dependent claim 55 contains a clerical oversight (reciting "method" instead of "kit"), although the claim is not subject to rejection on this ground. This can be remedied by amendment before issue.

§ 103(a) as allegedly obvious over Schroit in view of Gimbrone and Blankenberg. After Appellants responded to the rejections, the fifth Action withdrew the earlier § 102(e) rejection over Schroit. The fifth Action also withdrew the earlier § 103(a) rejection over Schroit, Gimbrone and Blankenberg, replacing this with the present § 103(a) rejection over Schroit, Gimbrone, Blankenberg and Abrams.

Thus, the Office has indicated that the pending claims are patentable over Schroit alone, and over Schroit, Gimbrone and Blankenberg in combination. Key questions to answer, therefore, are (1) whether the combination of Schroit, Gimbrone, Blankenberg and Abrams is proper; and (2) whether the addition of Abrams cures the lack of teaching or suggestion in Schroit, Gimbrone and Blankenberg. Appellants address these issues below, in addition to all aspects of the rejection.

2. The References have been Improperly Combined

Before the Office may combine the disclosure of two or more prior art references in order to establish a *prima facie* case of obviousness, there must be some teaching, suggestion or motivation to combine the references. *In re Rouffet*, 47 USPQ2d 1453, 1456 (Fed. Cir. 1998). Even if every element of an invention can be found in the prior art, obviousness is not established in the absence of sufficient "motivation to combine". *Rouffet* at 1457-1458. A high level of skill in the art cannot be held to substitute for the required motivation to combine. *Rouffet* at 1458.

In the present case, the references have been improperly combined, including the earlier improper combination of Schroit, Gimbrone and Blankenberg, and the new addition of Abrams.

Schroit, Gimbrone and Blankenberg were earlier said to be combinable because "each reference is directed to specific receptor molecule on the surface of human vascular endothelial cells associated with vascularized tumor" (fourth Action at page 5). Appellants traversed the

combination for reasons including that the references had been improperly characterized; the alleged motivation to combine was derived only from the present application and not from the cited art; and the combination was formulated with hindsight. The rejection was withdrawn.

The fifth Action adds Abrams to Schroit, Gimbrone and Blankenberg. Abrams is cited as teaching that "therapeutics [*sic*] conjugates directed to cancer cells comprising a targeting antibody and an active agent is [*sic*] well established in the art" (fifth Action at page 4). The alleged motivation to combine is said to be that the teachings of Schroit, Gimbrone, Blankenberg and Abrams "are in the same field of endeavor as they are all directed to the field of antibody immunology" (fifth Action at page 4). This is the only clear statement offered in an attempt to support the proposed combination of the four references.

The foregoing statement clearly shows that the references have been improperly combined. Blankenberg concerns <u>radiolabeled annexin</u> to image apoptosis and cell necrosis, mainly to detect inappropriate apoptosis, such as in neurons and the immune system, and also to detect insufficient apoptosis in tumor cells or virally infected cells (Blankenberg throughout, *e.g.*, column 5, lines 16-36 and column 12, lines 32-37). Blankenberg does <u>not</u> concern antibody immunology. As the field of antibody immunology provides the alleged motivation to combine, and as Blankenberg does not concern antibody immunology, the proposed combination is *prima facie* improper.

The fifth Action later returns to the combination of the "cited references" and again states that "each reference is directed to specific receptor molecule on the surface of human vascular endothelial cells associated with vascularized tumor" (fifth Action at page 8). In fact, <u>none</u> of the cited references concerns specific receptors on the surface of human vascular endothelial cells associated with vascularized tumors, which exist <u>only</u> in Appellants' specification. The

Action's statement therefore both improperly characterizes the references and is derived from the present application and not from the cited art, and is clearly improper. It is impermissible to use the claims as a frame and the prior-art references as a mosaic to piece together a facsimile of the claimed invention. *Uniroyal Inc. v. Rudkin-Wiley Corp.*, 5 USPQ2d 1434, 1438 (Fed. Cir. 1988); *W.L. Gore Assoc., Inc. v. Garlock, Inc.*, 220 USPQ 303, 312 (Fed. Cir. 1983).

Schroit concerns phosphatidylserine (PS) and reports that, as opposed to the situation in normal cells, PS may appear at the surface of tumor cells (Schroit throughout, *e.g.*, column 16, lines 27-33). Gimbrone concerns E-selectin expression on activated endothelium in certain diseases or infections, particularly in inflammation, and in connection with the metastatic spread of tumor cells (Gimbrone throughout, *e.g.*, abstract, column 4, line 57 to column 5, line 7). Blankenberg concerns radiolabeled annexin to image apoptosis and cell necrosis in neurons, the immune system, tumor cells and virally infected cells (Blankenberg throughout, *e.g.*, columns 5 and 12). Abrams concerns two or more different antibody species that react with different epitopes of a cancer cell (Abrams throughout, *e.g.*, abstract, claim 1).

Therefore, none of Schroit, Gimbrone, Blankenberg and Abrams teaches or suggests expression of PS, another aminophospholipid, E-selectin or any other specific receptor molecule on the surface of human vascular endothelial cells associated with a vascularized tumor. The statement in the fifth Action at page 8 is therefore in error and any additional reason for combining the cited references is improper. Resorting to the present application, rather than the cited references, also evidences "the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher", which renders both the combination and the resultant rejection improper. *W.L. Gore Assoc., Inc. v. Garlock, Inc.*, 220 USPQ 303, 313 (Fed. Cir. 1983).

In summary, Schroit, Gimbrone, Blankenberg and Abrams are not properly combinable because of their fundamentally disparate content and purposes. Schroit concerns the generation of PS-specific antibodies, which may bind to tumor cells; Gimbrone concerns treating diseases and infections, particularly inflammation, associated with E-selectin expression on activated endothelium; and Blankenberg concerns imaging apoptosis *in vivo* using radiolabeled annexin. Abrams, newly-cited, does not concern PS-specific antibodies, E-selectin or radiolabeled annexin, but rather relates to diagnostic or therapeutic agents attached to different antibodies that react with different epitopes of a cancer cell and have non-overlapping cross-reactivity.

The four cited references therefore have significantly different content and purposes and the references have been improperly combined. *In re Rouffet, supra.* The rejection is thus *prima facie* improper and should be reversed.

3. The References in Combination do Not Teach or Suggest the Claimed Invention, but Rather Teach Away from the Invention

The present claims are directed to kits comprising an aminophospholipid targeting agent-therapeutic agent construct, defined as "a first anti-cancer agent", and a second anti-cancer agent "other than" such a targeting agent-therapeutic agent construct, *i.e.*, a second, distinct anti-cancer agent (*e.g.*, claims 1 and 51). The targeting agents bind to aminophospholipids, such as PS (claim 3) or PE (claims 2 and 52), surprisingly found to be targetable markers on the luminal surface of tumor blood vessels (claim 49), and deliver the attached therapeutic agents to the tumor vasculature. The kits combine the targeting agent-therapeutic agent constructs with other anti-cancer agents (claims 27 and 28-31), including certain currently preferred anti-cancer agents (claims 53-57).

Even if prior art references have been properly combined, for an obviousness rejection to be proper under 35 U.S.C. § 103(a), it is required that the cited prior art suggest to those of

ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and that the prior art also convey to those of ordinary skill a reasonable expectation of success. *In re Dow Chemical Co.*, 5 USPQ 2d 1529, 1531 (Fed. Cir. 1988). Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in the applicant's disclosure. *Id.*

The proposed combination of four references is improper (see above). Even if properly combined, Schroit, Gimbrone, Blankenberg and Abrams in combination do not teach or suggest the claimed invention and do not provide the required reasonable expectation of success, but rather teach away from the claimed invention.

Schroit is cited as concerning techniques to prepare more specific antibodies and various types of antibodies (fifth Action at page 3). The fifth Action admits that Schroit fails to disclose "the use of other suitable antiphospholipid antibodies in combination with a second anticancer agent conjugated with a targeting antibody" (fifth Action at page 3). This statement, although distinguishing Schroit, is somewhat unclear. More precisely, Schroit does not teach or suggest an aminophospholipid targeting agent-therapeutic agent construct, and particularly fails to teach or suggest such a construct in combination with a second anti-cancer agent. Schroit's limitations in these areas are highlighted, at least in part, as the earlier § 102(e) rejection over Schroit was withdrawn following Appellants' response.

The Office first turned to Gimbrone and Blankenberg in an attempt to cure the deficiencies of Schroit, but withdrew the rejection. The fifth Action then added Abrams to Gimbrone and Blankenberg in a further attempt to cure the deficiencies of Schroit. Although Gimbrone, Blankenberg and Abrams have been improperly combined with Schroit, even if

properly combined, the four references together still fail to teach or suggest the kits of the present invention.

None of Gimbrone, Blankenberg and Abrams teaches or suggests an aminophospholipid targeting agent-therapeutic agent construct, let alone such a construct in combination with a second anti-cancer agent, and these references therefore fail to cure the deficiencies of Schroit. Gimbrone is silent as to aminophospholipids (fifth Action at page 4); Abrams is also silent as to aminophospholipids; and Blankenberg concerns only diagnostic constructs of annexin, without mention of a therapeutic agent (fifth Action at page 4). These deficiencies alone show the absence of a proper *prima facie* case of obviousness, or at least rebut any *prima facie* case that may have been made, and thus establish Appellant's entitlement to a patent. *Rouffet* at 1455.

In withdrawing the earlier rejection over Schroit, Gimbrone and Blankenberg, the fifth Action makes Abrams the focus of attention. As stated in the fifth Action at page 4, Abrams is clearly limited to antibody species that bind to <u>cancer cells</u> (Abrams at abstract; see also column 3, lines 51-68, particularly line 62). Aside from using two or more different antibody species of non-overlapping cross-reactivity, Abrams is generally representative of the field of immunotoxins that bind to cancer cells, known in the art prior to the present invention. Indeed, the background section of the present application states that immunotoxins for targeting cancer cells are known, but suffer from significant drawbacks (specification at pages 2-3). These drawbacks include survival of antigen-negative or antigen-deficient cells that can repopulate the tumor or lead to further metastases, and the fact that a solid tumor is generally impermeable to molecules of the size of antibodies and immunotoxins, such that the physical diffusion distances and the interstitial pressure within the tumor are significant limitations to therapy with immunotoxins against cancer cells (specification at page 3, lines 12-19).

Agents that treat solid tumors by targeting the tumor vasculature, as in the present invention, represent a significant and surprising advance over immunotoxins that bind to tumor cells, as represented by Abrams. Abrams does not teach or suggest any aspect of tumor vasculature targeting, but simply represents part of the state of the art prior to the invention. As Abrams concerns neither tumor vascular targeting nor aminophospholipids, Abrams is clearly irrelevant to the present invention and the rejection over Schroit, Gimbrone, Blankenberg and Abrams, even if properly combined, is overcome and should be reversed.

In reply to Appellants' earlier reasoning (addressing the rejection of Schroit, Gimbrone and Blankenberg, which was anyway withdrawn), the fifth Action at pages 6-8 discusses claim consturction [*sic*] in light of the case law and in regard to Schroit. The Action contends that Appellants' reasoning and the cited cases are not applicable as the claims do not exclude the teachings of the cited references, particularly Schroit, and as the cited references, particularly Schroit, do not teach away from the pending claims (fifth Action at page 6, with emphasis on Schroit at pages 7-8).

In response, the claims clearly exclude the teachings of Schroit as the § 102(e) rejection over Schroit has been withdrawn. Indeed, those aspects of the fifth Action (pages 7-8) that still question whether the present claims read on two aliquots of the first anti-cancer agent and allegedly "fall[s] within teachings of Schroit" are inconsistent with withdrawal of the § 102(e) rejection over Schroit.

The Action's position is simply that the phrase "other than" in the present claims means "the same as" (fifth Action at pages 7 and 8). In contrast, there is no way that one of ordinary skill in this art, or anyone versed in the English language, could interpret the claim language "a second anti-cancer agent <u>other than</u>" the first defined anti-cancer agent to mean two aliquots

of the same anti-cancer agent. Not only is "other than" not a synonym for "the same", it is essentially the opposite of "the same". Webster's collegiate dictionary defines "other" and "other than" as follows (**Exhibit D**):

> **Other**, being the one remaining or not included; being the one or ones distinct from that or those first mentioned or implied; in the every day sense, as "not the same: DIFFERENT" or "ADDITIONAL"; "one that remains of two or more"; "a thing opposite to or excluded by something else"; "a different or additional one"
>
> **Other than**, "with the exception of: EXCEPT FOR, BESIDES"
>
> Merriam-Webster's Collegiate Dictionary, Tenth Edition (capitals as in original), **Exhibit D**.

Therefore, one of ordinary skill would construe the pending claims, according to their "broadest reasonable interpretation", as the first defined anti-cancer agent and a second anti-cancer agent "other than, distinct from, not the same as, different to, in addition to, opposite to, excluding, with the exception of, except for or besides" the first anti-cancer agent. *In re Cortright,* 49 USPQ2d 1464, 1468 (Fed. Cir. 1999). In addition to *Cortright*, improperly dismissed by the Action, the foregoing claim construction is entirely consistent with *In re Morris*, 44 USPQ2d 1023, (Fed. Cir. 1997), cited in the fifth Action, which held:

> "The PTO applies to verbiage of the proposed claims the broadest reasonable meaning of the words in their ordinary usage as they would be understood by one of ordinary skill in the art, taking into account whatever enlightenment by way of definitions or otherwise that may be afforded by the written description contained in Appellants' specification."
>
> *In re Morris* at 1027; emphasis added.

The "broadest reasonable meaning" of "other than" cannot include "the same as". Accordingly, all pending claims, properly construed, clearly exclude the teachings of Schroit. Even if combined, Schroit, Gimbrone, Blankenberg and Abrams still fail to teach or suggest aminophospholipid targeting agent-therapeutic agent constructs, let alone such constructs in combination with other anti-cancer agent(s).

Schroit, Gimbrone, Blankenberg and Abrams, either alone or in any combination, also fail to teach or suggest targeting tumor vasculature, which exists only in Appellants' specification. The unexpected discovery that aminophospholipids are accessible, stable markers of tumor vasculature (specification throughout, *e.g.*, page 4, lines 24-26) underlies many surprising features of the claimed invention. This finding in itself was particularly surprising, as the tumor vascular endothelial cells are normal cells, taught in the prior art to preserve PS in the inner leaflet (*e.g.*, Schroit at column 16, lines 28-31; Blankenberg at column 5, lines 62-63).

The finding that aminophospholipids were markers of the normal cells of the tumor vasculature provided a means for effective therapy, overcoming the problems associated with tumor cell targeting, such as tumor cell resistance, antigen escape and effective penetration into the tumor (specification at pages 2-3). Moreover, the discovery of these aminophospholipid targets allows for the delivery of therapeutic agents in intimate contact with the tumor vascular endothelial cell membrane, allowing rapid entry into the target cell or rapid association with effector cells or components of the coagulation cascade (specification at page 5, lines 1-4).

Importantly, the translocation of aminophospholipids to the luminal surface of tumor vascular endothelial cells was further discovered to occur, at least in a significant part, independently of cell damage and apoptotic or other cell-death mechanisms (specification at page 5, lines 4-8). This discovery of sufficiently stable expression of aminophospholipids on morphologically intact tumor-associated vascular endothelial cells, in contrast to Schroit and Blankenberg, was an important step in the development of effective therapies (specification at page 5, lines 10-16).

Therefore, claims 1, 3-9, 19, 24-26, 50 and 51 are non-obvious over Schroit, Gimbrone, Blankenberg and Abrams, even if combined.

In addition, claims 45-48; claims 2 and 52; claims 16-18; claim 27; claims 28-31; claim 49; and claims 53-57 are even further removed from Schroit, Gimbrone, Blankenberg and Abrams, even in combination, as they recite further particular features not taught or suggested in the cited combination, as set forth herein. The cited references also include numerous examples of teaching away from the invention of claims 1, 3-9, 19, 24-26, 50 and 51, and even further examples of teaching away from the groups of claims that stand separately.

Schroit teaches away from the invention by teaching that PS does not appear at the surface of normal cells (Schroit throughout, *e.g.*, column 16, lines 27-33). This teaches away from the invention, which concerns aminophospholipid expression on normal cells, *i.e.*, tumor vascular endothelial cells (specification throughout, such as pages 4-5, page 68, lines 13-19; and claim 49). Targeting the normal endothelial cells of the blood vessels within a vascularized tumor has particular advantages, such as avoiding survival of antigen-negative or antigen-deficient tumor cells and overcoming the biophysical difficulties with access to the cancer cells (specification at page 3, lines 12-19).

Blankenberg also teaches away from the invention by teaching that PS resides only in the inner leaflet of normal cells and that the appearance of PS on the outer leaflet is indicative of apoptosis and cell death (Blankenberg throughout, *e.g.*, from column 5, line 62 to column 6, line 25). The present inventors not only surprisingly found that aminophospholipids were translocated to the luminal surface of normal tumor vascular endothelial cells, they unexpectedly found that this occurred, at least in a significant part, independently of apoptosis, cell damage and cell-death mechanisms. Thus, aminophospholipid surface expression on normal tumor vascular endothelial cells was found not to indicate cell death, in contrast to Blankenberg, but to

be sufficiently stable to allow tumor vasculature targeting and treatment (claim 49; specification throughout, *e.g.*, page 5, lines 1-16; and page 68, lines 13-19).

Schroit further teaches away from claims 2 and 49, drawn to kits in which the targeting agent binds to phosphatidylethanolamine (PE). The objective of Schroit is to produce highly-specific anti-PS antibodies (Schroit at column 2, line 36), which are said to recognize PS but not DPOE (dioleoyl phosphatidylethanolamine) in a bilayer membrane (Schroit at column 25, lines 24-26). By teaching the desirability and generation of antibodies that do not bind to PE, Schroit emphatically teaches away from claims 2 and 49, drawn to kits in which the targeting agent does bind to PE.

None of Schroit, Gimbrone, Blankenberg and Abrams, either alone or in any combination, teaches or suggests a targeting agent-therapeutic agent construct in which the targeting agent is operatively attached to a coagulant, such as Tissue Factor or a Tissue Factor derivative. Claims 16-18, which are directed to these embodiments, are thus even further removed from Schroit, Gimbrone, Blankenberg and Abrams, even in combination.

To the extent that the Office deems the anti-E-selectin conjugates of Gimbrone to be relevant, Gimbrone teaches away from claims 16-18, directed to kits in which the attached therapeutic agent is a coagulant, such as a Tissue Factor derivative, *e.g.*, truncated Tissue Factor. Gimbrone teaches away from these aspects of the invention by disclosing an anti-coagulant immunoconjugate (Gimbrone at Example 5), rather than a coagulant immunoconjugate.

Schroit, Gimbrone, Blankenberg and Abrams, either alone or in any combination, fail to teach or suggest a targeting agent-therapeutic agent construct that binds to an aminophospholipid in combination with any second, distinct anti-cancer agent. Claim 27, which specifies that the second anti-cancer agent is a chemotherapeutic agent, radiotherapeutic agent, anti-angiogenic

agent or apoptosis-inducing agent, is even further removed from Schroit, Gimbrone, Blankenberg and Abrams, which separately and together particularly fail to teach or suggest combinations with such second anti-cancer agents.

Claims 28-31, which recite aminophospholipid targeting agent-therapeutic agent constructs in combination with a second anti-cancer agent in the form of an immunoconjugate that binds to tumor cells, stroma or vasculature, are also even further removed from Schroit, Gimbrone, Blankenberg and Abrams, even in combination, which do not teach or suggest combinations with any such second anti-cancer agents.

The present invention, unlike the cited prior art, also provides for the intelligent selection of aminophospholipid targeting agent-therapeutic agent constructs and second anti-cancer agents in a kit for use together, such as for simultaneous or sequential administration to an animal with cancer (*e.g.*, specification at pages 39-40 and 151-173). These embodiments are represented in claims 53-57, which are directed to therapeutic kits in which the second anti-cancer agents increase aminophospholipid expression, injure or induce apoptosis in tumor blood vessel endothelium, or that kill or inhibit the metastasis of tumor cells. None of Schroit, Gimbrone, Blankenberg and Abrams, either alone or in combination, teaches or suggests such anti-cancer agents as the second component in an aminophospholipid targeting kit of the claimed invention. Claims 53-57 are thus even further distanced from the cited art.

In contrast to the cited prior art, the present application teaches the rationale for selecting second anti-cancer agents, along with detailed teaching concerning anti-cancer agents within each category. These aspects of the invention are derived, in part, from the inventors' discovery that factors and conditions in the tumor microenvironment cause aminophospholipid

translocation in <u>normal</u> endothelial cells (specification throughout, *e.g.*, Example XIV; see also, Exhibit A to Appellants' fourth response[4]).

Exhibit A to Appellants' fourth response and a related article subsequently published on behalf of the inventors (Ran *et al., Cancer Res.*, 62:6132-40, 2002) are attached hereto as **Exhibit E**. The data in **Exhibit E** further support the inventors' surprising findings, as set forth in the specification, showing that factors and conditions in tumors interact to give amplified effects on aminophospholipid exposure on the normal tumor vascular endothelial cells. This surprising information, which was applied in inventing the kits of the present claims, exists in the present application but not in the cited art. From such information, aminophospholipid targeting agent-therapeutic agent constructs and particular second anti-cancer agents can now be selected for advantageous use together (*e.g.*, specification at pages 39-40, 151-173 and Example XIV). Such selections include those in claims 53-57, which are thus even further removed from Schroit, Gimbrone, Blankenberg and Abrams, both alone and in combination.

In summary, Schroit, Gimbrone, Blankenberg and Abrams have been improperly combined, and even if combined, do not teach or suggest the claimed invention or provide the reasonable expectation of success necessary to render the claims legally obvious. The references also teach away from various aspects of the claimed invention. The § 103(a) rejection is in error and should be reversed. *In re Rouffet, supra; In re Dow Chemical Co., supra.*

For the foregoing reasoning, the § 103(a) rejection of claims 1-9, 16-19, 24-31 and 45-57 is in error and should be reversed.

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[4]The fifth Action improperly ignored Exhibit A, stating only that it was not persuasive as being "directed to antibodies that are not claimed here" (fifth Action at page 8).

B. Whether a Proper Provisional Rejection for Obviousness-Type Double Patenting has been Established for Claims 1-9, 16-19, 24-32, 43 and 45-49 Over the '862 Application

The fifth Action provisionally rejects claims 1-9, 16-19, 24-32, 43 and 45-49 under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over "pending claim" of the '862 application (Attorney Docket No. 4001.002282). Claims 50-57 are excluded from this ground of rejection. This is said to be a "one-way obviousess [*sic*] double patenting rejection" (fifth Action at page 6).

Appellants question, on several grounds, whether a proper provisional obviousness-type double patenting rejection has been established[5].

Firstly, and most importantly, the prosecution history of the present application indicates a two-way obviousness double patenting rejection. For example, the third Action states that the claims in the two applications "are not patentably distinct from each other" (third Action at page 10; emphasis added). The fourth Action maintains this, stating that the claims "are not patentably distinct from each other" and that the claims "overlap" (fourth Action at pages 5 and 6; emphasis added). Even the fifth Action still states that the claims are "not patentably distinct from each other" and refers to the "overlapping nature of the claimed subject matter" (fifth Action at page 5; emphasis added). If the claims in the two applications "are not patentably distinct from each other", as stated in the third, fourth and fifth Actions, the present rejection cannot be proper without a provisional or actual obviousness-type double patenting rejection in the '862 application over the claims of the present application.

[5]Also, the present claims are not generic to the claims in the '862 application (fifth Action at page 5). In the present kit claims, the aminophospholipid targeting agent is operatively attached to a therapeutic agent. The kits of the '862 application claim an unconjugated anti-aminophospholipid antibody. Unconjugated antibodies are not species within the genus of targeting agent-therapeutic agent conjugates.

Secondly, and consistent with the statements of two-way obviousness double patenting in this prosecution history, the discussion of provisional obviousness-type double patenting rejections between co-pending applications in the MPEP is limited to entering the double patenting rejection in <u>each</u> of the co-pending applications. MPEP 804 (Eighth Edition at page 800-19). Appellants therefore believe that for the present rejection to be proper, a provisional or actual double patenting rejection should be entered in the '862 application.

Thirdly, although one-way obviousness double patenting rejections have judicial authority, it seems that such rejections require comparison of a pending application to an issued patent. For example, contrast the discussion of co-pending applications at MPEP page 800-19 with the discussion of one-way obviousness at MPEP page 800-23, which relates to an application and a patent.

Appellants therefore seek clarification of whether the provisional obviousness-type double patenting rejection is proper in the absence of a counterpart rejection in the '862 application.

In any event, should the double patenting rejection be maintained and become the only remaining rejection in the case, Appellants will submit a Terminal Disclaimer to overcome the rejection.

For the foregoing reasoning, the provisional obviousness-type double patenting rejection of claims 1-9, 16-19, 24-32, 43 and 45-49 is either improper or will be moot upon submission of a Terminal Disclaimer.

IX. CONCLUSION

In view of the foregoing reasoning, Appellants submit that the outstanding rejection is unwarranted and respectfully request that the Board of Patent Appeals and Interferences reverse the rejection of claims 1-9, 16-19, 24-31 and 45-57 and direct the Examiner to pass the case to

issue. More particularly, that the Board find claims 1-9, 16-19, 24-31 and 45-57 to be non-obvious under 35 U.S.C. § 103(a) over Schroit, Gimbrone, Blankenberg and Abrams; and that the Board determine whether the provisional obviousness-type double patenting rejection over the '862 application is proper, and if proper, direct the Examiner to permit Appellants to enter a Terminal Disclaimer.

Respectfully submitted,
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APPENDIX A
CLAIMS ON APPEAL
SERIAL NO. 09/351,149 (3999.002383)

1. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, biologically effective amounts of at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent; and:

   (a) a targeting agent-detectable agent construct that comprises a second targeting agent that binds to an aminophospholipid operatively attached to a detectable agent; or

   (b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

2. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises a targeting agent that binds to phosphatidylethanolamine.

3. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises a targeting agent that binds to phosphatidylserine.

4. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises at least a first anti-aminophospholipid antibody or antigen-binding fragment thereof.

5. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first IgG or IgM anti-aminophospholipid antibody.

6. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding fragment of an anti-aminophospholipid antibody.

7. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first recombinant anti-aminophospholipid antibody, or antigen-binding fragment thereof.

8. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first human, humanized or part-human chimeric anti-aminophospholipid antibody, or antigen-binding fragment thereof.

9. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first monoclonal anti-aminophospholipid antibody, or antigen-binding fragment thereof.

16. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises at least a first coagulant.

17. (Original) The kit of claim 16, wherein said targeting agent-therapeutic agent construct comprises at least a first Tissue Factor, dimeric Tissue Factor, trimeric Tissue Factor, polymeric Tissue Factor, mutant Tissue Factor, truncated Tissue Factor or a Tissue Factor derivative.

18. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

19. (Original) The kit of claim 1, wherein said kit comprises at least a first pharmaceutically acceptable formulation suitable for intravenous administration.

24. (Original) The kit of claim 1, wherein said kit comprises said at least a first targeting agent-therapeutic agent construct in combination with said at least a second anti-cancer agent.

25. (Original) The kit of claim 24, wherein said at least a first targeting agent-therapeutic agent construct and said at least a second anti-cancer agent are comprised within a single pharmaceutical composition.

26. (Original) The kit of claim 24, wherein said at least a first targeting agent-therapeutic agent construct and said at least a second anti-cancer agent are comprised within distinct pharmaceutical compositions.

27. (Original) The kit of claim 24, wherein said at least a second anti-cancer agent is a chemotherapeutic agent, radiotherapeutic agent, anti-angiogenic agent or apoptosis-inducing agent.

28. (Original) The kit of claim 24, wherein said at least a second anti-cancer agent is an antibody-therapeutic agent construct comprising a second targeting antibody, or antigen-binding fragment thereof, that binds to a surface-expressed, surface-accessible or surface-localized component of a tumor cell, tumor stroma or tumor vasculature; wherein said targeting antibody or fragment thereof is operatively linked to a therapeutic agent.

29. (Original) The kit of claim 28, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed, surface-accessible, surface-localized, cytokine-inducible or coagulant-inducible component of intratumoral blood vessels of a vascularized tumor.

30. (Original) The kit of claim 29, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a component of intratumoral vasculature selected from the group consisting of an aminophospholipid, endoglin, a TGFβ receptor, E-selectin, P-selectin, VCAM-1, ICAM-1, PSMA, a VEGF/VPF receptor, an FGF receptor, a TIE, $\alpha_v\beta_3$ integrin, pleiotropin, endosialin, an MHC Class II protein, VEGF/VPF, FGF, TGFβ, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor/hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF and TIMP.

31. (Original) The kit of claim 28, wherein said second targeting antibody, or antigen-binding fragment thereof, is operatively linked to gelonin, deglycosylated ricin A chain, Tissue Factor, truncated Tissue Factor or to an antibody, or antigen-binding fragment thereof, that binds to Tissue Factor or truncated Tissue Factor.

32. (Previously Presented) The kit of claim 1, wherein said kit comprises biologically effective amounts of:

(a) said at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent;

(b) said targeting agent-detectable agent construct that comprises a second targeting agent that binds to an aminophospholipid operatively attached to a detectable agent; and

(c) said at least a second anti-cancer agent.

Claims 33-42 cancelled

43. (Previously Presented) In combination, biologically effective amounts of:

(a) a first composition comprising at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent;

(b) a second composition comprising a targeting agent-detectable agent construct that comprises a second targeting agent that binds to an aminophospholipid operatively attached to a detectable agent; and

(c) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

45. (Previously Presented) The combination of claim 43, wherein said first composition is a pharmaceutical composition.

46. (Previously Presented) The combination of claim 43, wherein said second composition is a pharmaceutical composition.

47. (Previously Presented) The combination of claim 43, wherein said at least a second anti-cancer agent is admixed with said at least a first targeting agent-therapeutic agent construct to form a therapeutic cocktail.

48. (Previously Presented) The combination of claim 43, wherein said at least a second anti-cancer agent is comprised within a composition distinct from said at least a first targeting agent-therapeutic agent construct.

49. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, biologically effective amounts of at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent, wherein said at least a first targeting agent binds to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor; and

(a) a targeting agent-detectable agent construct that comprises a second targeting agent operatively attached to a detectable agent, wherein said second targeting agent binds to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor; or

(b) an anti-cancer agent.

50. (Previously Presented) The kit of claim 1, wherein said kit comprises at least a first pharmaceutically acceptable liposomal formulation.

51. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, therapeutically effective amounts of:

(a) at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent; and

(b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

52. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, therapeutically effective amounts of:

(a) at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent, wherein said at least a first targeting agent binds to phosphatidylethanolamine expressed on the luminal surface of blood vessels of a vascularized tumor; and

(b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

53. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, therapeutically effective amounts of:

(a) at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent, wherein said at least a first targeting agent binds to an aminophospholipid on the luminal surface of blood vessels of a vascularized tumor; and

(b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct; wherein said at least a second anti-cancer agent:

(i) increases aminophospholipid expression in the endothelium of said blood vessels of said vascularized tumor or injures or induces apoptosis in the endothelium of said blood vessels of said vascularized tumor; or

(ii) kills tumor cells of said tumor or is an anti-angiogenic agent that inhibits metastasis of tumor cells.

54. (Previously Presented) The kit of claim 53, wherein said at least a second anti-cancer agent increases aminophospholipid expression in the endothelium of said blood vessels of said vascularized tumor or injures or induces apoptosis in the endothelium of said blood vessels of said vascularized tumor.

55. (Previously Presented) The method of claim 54, wherein said at least a second anti-cancer agent is taxol, vincristine, vinblastine, neomycin, a combretastatin, a podophyllotoxin, TNF-α, angiostatin, endostatin, vasculostatin, an $\alpha_v\beta_3$ antagonist, a calcium-flux inducing agent, a calcium ionophore, H_2O_2, thrombin, an inflammatory cytokine or interleukin-4.

56. (Previously Presented) The kit of claim 53, wherein said at least a second anti-cancer agent kills tumor cells of said tumor or is an anti-angiogenic agent that inhibits metastasis of tumor cells.

57. (Previously Presented) The kit of claim 56, wherein said at least a second anti-cancer agent is an anti-tumor cell immunoconjugate, a chemotherapeutic agent or an anti-angiogenic agent.

APPENDIX B
PENDING CLAIMS AT APPEAL
SERIAL NO. 09/351,149 (3999.002383)

1. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, biologically effective amounts of at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent; and:

(a) a targeting agent-detectable agent construct that comprises a second targeting agent that binds to an aminophospholipid operatively attached to a detectable agent; or

(b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

2. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises a targeting agent that binds to phosphatidylethanolamine.

3. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises a targeting agent that binds to phosphatidylserine.

4. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises at least a first anti-aminophospholipid antibody or antigen-binding fragment thereof.

5. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first IgG or IgM anti-aminophospholipid antibody.

6. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding fragment of an anti-aminophospholipid antibody.

7. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first recombinant anti-aminophospholipid antibody, or antigen-binding fragment thereof.

8. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first human, humanized or part-human chimeric anti-aminophospholipid antibody, or antigen-binding fragment thereof.

9. (Original) The kit of claim 4, wherein said targeting agent-therapeutic agent construct comprises at least a first monoclonal anti-aminophospholipid antibody, or antigen-binding fragment thereof.

10. (Pending, but withdrawn) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises at least a first aminophospholipid binding protein or an aminophospholipid-binding fragment thereof.

11. (Pending, but withdrawn) The kit of claim 10, wherein said targeting agent-therapeutic agent construct comprises at least a first phosphatidylserine binding protein or a phosphatidylserine-binding fragment thereof.

12. (Pending, but withdrawn) The kit of claim 10, wherein said targeting agent-therapeutic agent construct comprises at least a first phosphatidylethanolamine binding protein or a phosphatidylethanolamine-binding fragment thereof.

13. (Pending, but withdrawn) The kit of claim 10, wherein said targeting agent-therapeutic agent construct comprises at least a first Annexin V or kininogen or an aminophospholipid-binding fragment thereof.

14. (Pending, but withdrawn) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises at least a first anticellular or cytotoxic agent.

15. (Pending, but withdrawn) The kit of claim 14, wherein said targeting agent-therapeutic agent construct comprises at least a first gelonin, ricin A chain or deglycosylated ricin A chain.

16. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises at least a first coagulant.

17. (Original) The kit of claim 16, wherein said targeting agent-therapeutic agent construct comprises at least a first Tissue Factor, dimeric Tissue Factor, trimeric Tissue Factor, polymeric Tissue Factor, mutant Tissue Factor, truncated Tissue Factor or a Tissue Factor derivative.

18. (Original) The kit of claim 1, wherein said targeting agent-therapeutic agent construct comprises an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

19. (Original) The kit of claim 1, wherein said kit comprises at least a first pharmaceutically acceptable formulation suitable for intravenous administration.

20. (Pending, but withdrawn) The kit of claim 1, wherein said kit comprises, in distinct pharmaceutical compositions, said at least a first targeting agent-therapeutic agent construct in combination with said targeting agent-detectable agent construct.

21. (Pending, but withdrawn) The kit of claim 20, wherein said targeting agent-detectable agent construct comprises the X-ray detectable compound bismuth (III), gold (III), lanthanum (III) or lead (II).

22. (Pending, but withdrawn) The kit of claim 20, wherein said targeting agent-detectable agent construct comprises the detectable radioactive ion copper^{67}, gallium^{67}, gallium^{68}, indium^{111}, indium^{113}, iodine^{123}, iodine^{125}, iodine^{131}, mercury^{197}, mercury^{203}, rhenium^{186}, rhenium^{188}, rubidium^{97}, rubidium^{103}, technetium^{99m} or yttrium^{90}.

23. (Pending, but withdrawn) The kit of claim 20, wherein said targeting agent-detectable agent construct comprises the detectable nuclear magnetic spin-resonance isotope cobalt (II), copper (II), chromium (III), dysprosium (III), erbium (III), gadolinium (III), holmium (III), iron

(II), iron (III), manganese (II), neodymium (III), nickel (II), samarium (III), terbium (III), vanadium (II) or ytterbium (III).

24. (Original) The kit of claim 1, wherein said kit comprises said at least a first targeting agent-therapeutic agent construct in combination with said at least a second anti-cancer agent.

25. (Original) The kit of claim 24, wherein said at least a first targeting agent-therapeutic agent construct and said at least a second anti-cancer agent are comprised within a single pharmaceutical composition.

26. (Original) The kit of claim 24, wherein said at least a first targeting agent-therapeutic agent construct and said at least a second anti-cancer agent are comprised within distinct pharmaceutical compositions.

27. (Original) The kit of claim 24, wherein said at least a second anti-cancer agent is a chemotherapeutic agent, radiotherapeutic agent, anti-angiogenic agent or apoptosis-inducing agent.

28. (Original) The kit of claim 24, wherein said at least a second anti-cancer agent is an antibody-therapeutic agent construct comprising a second targeting antibody, or antigen-binding fragment thereof, that binds to a surface-expressed, surface-accessible or surface-localized component of a tumor cell, tumor stroma or tumor vasculature; wherein said targeting antibody or fragment thereof is operatively linked to a therapeutic agent.

29. (Original) The kit of claim 28, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed, surface-accessible, surface-localized, cytokine-inducible or coagulant-inducible component of intratumoral blood vessels of a vascularized tumor.

30. (Original) The kit of claim 29, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a component of intratumoral vasculature selected from the group consisting of an aminophospholipid, endoglin, a TGFβ receptor, E-selectin, P-selectin, VCAM-1, ICAM-1, PSMA, a VEGF/VPF receptor, an FGF receptor, a TIE, $\alpha_v\beta_3$ integrin, pleiotropin, endosialin, an MHC Class II protein, VEGF/VPF, FGF, TGFβ, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor/hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF and TIMP.

31. (Original) The kit of claim 28, wherein said second targeting antibody, or antigen-binding fragment thereof, is operatively linked to gelonin, deglycosylated ricin A chain, Tissue Factor, truncated Tissue Factor or to an antibody, or antigen-binding fragment thereof, that binds to Tissue Factor or truncated Tissue Factor.

32. (Objected To) The kit of claim 1, wherein said kit comprises biologically effective amounts of:

(a) said at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent;

(b) said targeting agent-detectable agent construct that comprises a second targeting agent that binds to an aminophospholipid operatively attached to a detectable agent; and

(c) said at least a second anti-cancer agent.

Claims 33-42 cancelled

43. (Allowed) In combination, biologically effective amounts of:

(a) a first composition comprising at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent;

(b) a second composition comprising a targeting agent-detectable agent construct that comprises a second targeting agent that binds to an aminophospholipid operatively attached to a detectable agent; and

(c) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

44. (Pending, but withdrawn) The kit of claim 20, wherein the targeting agent of said at least a first targeting agent-therapeutic agent construct and the targeting agent of said targeting agent-detectable agent construct are anti-aminophospholipid antibodies, or antigen-binding fragments thereof, obtained from the same antibody preparation or antibody-producing hybridoma.

45. (Previously Presented) The combination of claim 43, wherein said first composition is a pharmaceutical composition.

46. (Previously Presented) The combination of claim 43, wherein said second composition is a pharmaceutical composition.

47. (Previously Presented) The combination of claim 43, wherein said at least a second anti-cancer agent is admixed with said at least a first targeting agent-therapeutic agent construct to form a therapeutic cocktail.

48. (Previously Presented) The combination of claim 43, wherein said at least a second anti-cancer agent is comprised within a composition distinct from said at least a first targeting agent-therapeutic agent construct.

49. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, biologically effective amounts of at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent, wherein said at least a first targeting agent binds to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor; and

(a) a targeting agent-detectable agent construct that comprises a second targeting agent operatively attached to a detectable agent, wherein said second targeting agent binds to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor; or

(b) an anti-cancer agent.

50. (Previously Presented) The kit of claim 1, wherein said kit comprises at least a first pharmaceutically acceptable liposomal formulation.

51. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, therapeutically effective amounts of:

(a) at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent; and

(b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

52. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, therapeutically effective amounts of:

(a) at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent, wherein said at least a first targeting agent binds to phosphatidylethanolamine expressed on the luminal surface of blood vessels of a vascularized tumor; and

(b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct.

53. (Previously Presented) A kit comprising, in a pharmaceutically acceptable form, therapeutically effective amounts of:

(a) at least a first anti-cancer agent, wherein said at least a first anti-cancer agent is at least a first targeting agent-therapeutic agent construct that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent, wherein said at least a first targeting agent binds to an aminophospholipid on the luminal surface of blood vessels of a vascularized tumor; and

(b) at least a second anti-cancer agent other than said at least a first targeting agent-therapeutic agent construct; wherein said at least a second anti-cancer agent:

(i) increases aminophospholipid expression in the endothelium of said blood vessels of said vascularized tumor or injures or induces apoptosis in the endothelium of said blood vessels of said vascularized tumor; or

(ii) kills tumor cells of said tumor or is an anti-angiogenic agent that inhibits metastasis of tumor cells.

54. (Previously Presented) The kit of claim 53, wherein said at least a second anti-cancer agent increases aminophospholipid expression in the endothelium of said blood vessels of said vascularized tumor or injures or induces apoptosis in the endothelium of said blood vessels of said vascularized tumor.

55. (Previously Presented) The method of claim 54, wherein said at least a second anti-cancer agent is taxol, vincristine, vinblastine, neomycin, a combretastatin, a podophyllotoxin, TNF-α, angiostatin, endostatin, vasculostatin, an $\alpha_v\beta_3$ antagonist, a calcium-flux inducing agent, a calcium ionophore, H_2O_2, thrombin, an inflammatory cytokine or interleukin-4.

56. (Previously Presented) The kit of claim 53, wherein said at least a second anti-cancer agent kills tumor cells of said tumor or is an anti-angiogenic agent that inhibits metastasis of tumor cells.

57. (Previously Presented) The kit of claim 56, wherein said at least a second anti-cancer agent is an anti-tumor cell immunoconjugate, a chemotherapeutic agent or an anti-angiogenic agent.

EXHIBIT A
ALLOWED CLAIMS
APPLICATION SERIAL NO. 09/351,598

1. A binding ligand comprising at least a first targeting agent that binds to an aminophospholipid operatively attached to at least a first therapeutic agent.

2. The binding ligand of claim 1, wherein said targeting agent binds to phosphatidylethanolamine.

3. The binding ligand of claim 1, wherein said targeting agent binds to phosphatidylserine.

4. The binding ligand of claim 1, wherein said targeting agent comprises at least a first anti-aminophospholipid antibody or antigen-binding fragment thereof.

5. The binding ligand of claim 4, wherein said targeting agent comprises at least a first IgG or IgM anti-aminophospholipid antibody.

6. The binding ligand of claim 4, wherein said targeting agent comprises at least a first scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding region of an anti-aminophospholipid antibody.

7. The binding ligand of claim 4, wherein said targeting agent comprises at least a first recombinant anti-aminophospholipid antibody or antigen-binding fragment thereof.

8. The binding ligand of claim 4, wherein said targeting agent comprises at least a first human, humanized or part-human chimeric anti-aminophospholipid antibody or antigen-binding fragment thereof.

9. The binding ligand of claim 8, wherein said targeting agent comprises at least a first recombinant, human anti-aminophospholipid antibody or antigen-binding fragment thereof.

10. The binding ligand of claim 8, wherein said targeting agent comprises at least a first anti-aminophospholipid antibody in which a mouse antibody variable region that binds to an aminophospholipid is operatively attached to a human antibody framework or constant region.

11. The binding ligand of claim 4, wherein said targeting agent comprises at least a first dimer, trimer or multimer of an anti-aminophospholipid antibody or antigen-binding fragments thereof.

12. The binding ligand of claim 4, wherein said targeting agent comprises at least a first monoclonal anti-aminophospholipid antibody or antigen-binding fragment thereof.

13. The binding ligand of claim 12, wherein monoclonal anti-aminophospholipid antibody, or antigen-binding fragment thereof, is obtained from a human patient that has a disease associated with the production of anti-aminophospholipid antibodies.

14. The binding ligand of claim 12, wherein said monoclonal anti-aminophospholipid antibody, or antigen-binding fragment thereof, is obtained by a process comprising stimulating a mixed population of human peripheral blood lymphocytes with an immunogenically effective amount of an aminophospholipid sample.

15. The binding ligand of claim 12, wherein said monoclonal anti-aminophospholipid antibody, or antigen-binding fragment thereof, is obtained by a process comprising immunizing an animal with an immunogenically effective amount of an aminophospholipid sample.

16. The binding ligand of claim 15, wherein the immunized animal is a transgenic mouse that comprises a human antibody library and wherein the anti-aminophospholipid monoclonal antibody is a human monoclonal antibody.

17. The binding ligand of claim 12, wherein said monoclonal anti-aminophospholipid antibody, or antigen-binding fragment thereof, is obtained by a process comprising isolating and expressing an anti-aminophospholipid antibody-encoding nucleic acid from an anti-aminophospholipid antibody-producing cell.

18. The binding ligand of claim 1, wherein said targeting agent comprises at least a first aminophospholipid binding protein or an aminophospholipid-binding fragment thereof.

19. The binding ligand of claim 18, wherein said targeting agent comprises at least a first annexin or a phosphatidylserine-binding fragment thereof.

20. The binding ligand of claim 19, wherein said targeting agent comprises at least a first Annexin V or a phosphatidylserine-binding fragment thereof.

21. The binding ligand of claim 18, wherein said targeting agent comprises at least a first phosphatidylethanolamine binding protein or a phosphatidylethanolamine-binding fragment thereof.

22. The binding ligand of claim 21, wherein said targeting agent comprises at least a first kininogen or a phosphatidylethanolamine-binding fragment thereof.

23. The binding ligand of claim 1, wherein said targeting agent comprises at least two aminophospholipid binding sites.

24. The binding ligand of claim 1, wherein said targeting agent is attached to at least a first anticellular or cytotoxic agent.

25. The binding ligand of claim 24, wherein said targeting agent is attached to at least a first steroid, cytokine, antimetabolite, anthracycline, vinca alkaloid, antibiotic, alkylating agent, epipodophyllotoxin, DNA synthesis inhibitor, daunorubicin, doxorubicin or adriamycin.

26. The binding ligand of claim 24, wherein said targeting agent is attached to at least a first plant-, fungus- or bacteria-derived toxin.

27. The binding ligand of claim 26, wherein said targeting agent is attached to at least a first A chain toxin, bacterial endotoxin, lipid A moiety of bacterial endotoxin, ribosome inactivating protein, α-sarcin, gelonin, aspergillin, restrictocin, ribonuclease, diphtheria toxin or *Pseudomonas* exotoxin.

28. The binding ligand of claim 27, wherein said targeting agent is attached to at least a first ricin A chain or deglycosylated ricin A chain.

29. The binding ligand of claim 1, wherein said targeting agent is attached to at least a first coagulant.

30. The binding ligand of claim 29, wherein said targeting agent is attached to at least a first human coagulation factor.

31. The binding ligand of claim 29, wherein said targeting agent is attached to at least a first coagulation factor selected from the group consisting of Factor II/IIa, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, a vitamin K-dependent coagulation factor that lacks the Gla modification, Russell's viper venom Factor X activator, thromboxane A_2, thromboxane A_2 synthase and α2-antiplasmin.

32. The binding ligand of claim 29, wherein said targeting agent is attached to at least a first Tissue Factor, dimeric Tissue Factor, trimeric Tissue Factor, polymeric Tissue Factor, mutant Tissue Factor or Tissue Factor derivative.

33. The binding ligand of claim 32, wherein said targeting agent is attached to at least a first truncated Tissue Factor.

34. The binding ligand of claim 1, wherein said targeting agent is directly attached to said therapeutic agent by a direct covalent bond, via a chemical cross-linker or by recombinant expression as a fusion protein.

35. The binding ligand of claim 1, wherein said targeting agent is indirectly attached to said therapeutic agent via an antibody, or antigen binding region thereof, that binds to said therapeutic agent.

36. The binding ligand of claim 1, wherein said targeting agent is attached to at least two distinct therapeutic agents.

37. The binding ligand of claim 1, wherein said targeting agent is an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

38. The binding ligand of claim 1, dispersed in a pharmaceutically acceptable formulation.

39. A binding ligand comprising at least a first targeting agent operatively attached to a therapeutic agent; wherein said targeting agent binds to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor.

40. An antibody construct, comprising at least a first anti-aminophospholipid antibody, or antigen-binding fragment thereof, operatively attached to at least a first therapeutic agent.

41. The antibody construct of claim 40, comprising at least a first anti- phosphatidylserine antibody, or antigen-binding fragment thereof.

42. The antibody construct of claim 40, wherein said at least a first anti-aminophospholipid antibody, or antigen-binding fragment thereof, is operatively attached to Tissue Factor, truncated Tissue Factor or a Tissue Factor derivative.

43. The antibody construct of claim 40, wherein said antibody construct is a bispecific antibody construct.

44. A bispecific antibody, comprising a first antigen-binding region that binds to an aminophospholipid operatively attached to a second antigen-binding region that binds to a therapeutic agent.

45. The bispecific antibody of claim 44, comprising a first antigen-binding region that binds to phosphatidylserine.

46. The bispecific antibody of claim 44, comprising a second antigen-binding region that binds to Tissue Factor, truncated Tissue Factor or a Tissue Factor derivative.

47. The bispecific antibody of claim 46, further comprising Tissue Factor, truncated Tissue Factor or a Tissue Factor derivative bound to said second antigen-binding region.

48. An anti-phosphatidylserine antibody construct, comprising an anti-phosphatidylserine antibody, or antigen-binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

49. A binding construct, comprising at least a first aminophospholipid binding protein, or aminophospholipid-binding fragment thereof, operatively attached to at least a first therapeutic agent.

50. The binding construct of claim 49, comprising at least a first annexin or a phosphatidylserine-binding fragment thereof.

51. The binding construct of claim 49, comprising at least a first aminophospholipid binding protein, or aminophospholipid-binding fragment thereof, operatively attached to Tissue Factor, truncated Tissue Factor or a Tissue Factor derivative.

52. An annexin conjugate, comprising Annexin V operatively attached to truncated Tissue Factor.

53. A composition comprising at least a first binding ligand that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent; wherein said targeting agent binds to an aminophospholipid.

54. The composition of claim 53, wherein said composition comprises a binding ligand that comprises an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

55. The composition of claim 53, wherein said composition comprises at least a first and second binding ligand that each bind to an aminophospholipid.

56. The composition of claim 55, wherein said composition comprises at least a first binding ligand that binds to phosphatidylethanolamine and at least a second binding ligand that binds to phosphatidylserine.

57. The composition of claim 53, wherein said composition comprises at least a second anti-cancer agent.

58. The composition of claim 53, wherein said composition is a pharmaceutically acceptable composition.

59. A pharmaceutical composition comprising at least a first binding ligand that comprises at least a first targeting agent operatively attached to at least a first therapeutic agent; wherein said targeting agent localizes to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor upon administration of said pharmaceutical composition to an animal with a vascularized tumor.

60. The pharmaceutical composition of claim 59, wherein said composition comprises a binding ligand that comprises an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

61. The pharmaceutical composition of claim 59, wherein said composition comprises at least a second anti-cancer agent.

62. The pharmaceutical composition of claim 59, wherein said at least a second anti-cancer agent is a chemotherapeutic agent, radiotherapeutic agent, anti-angiogenic agent or apoptosis-inducing agent.

63. The pharmaceutical composition of claim 59, wherein said at least a second anti-cancer agent is an antibody-therapeutic agent construct comprising a targeting antibody, or antigen-binding fragment thereof, that binds to a surface-expressed, surface-accessible or surface-localized component of a tumor cell, tumor stroma or tumor vasculature; wherein said targeting antibody or fragment thereof is operatively linked to a therapeutic agent.

64. The binding ligand of claim 38, formulated as a liposomal formulation.

EXHIBIT B
ISSUED CLAIMS
PATENT NO. 6,312,694 (SERIAL NO. 09/351,457)

1. (Amended) A method for killing tumor vascular endothelial cells, comprising administering to an animal having a vascularized tumor a biologically effective amount of at least a first binding ligand; wherein said binding ligand comprises a selected cytotoxic agent operatively attached to a targeting agent and wherein said targeting agent binds to an aminophospholipid on the luminal surface of tumor vascular endothelial cells.

2. (Amended) A method for inducing tumor vasculature coagulation, comprising administering to an animal having a vascularized tumor a vessel-occluding amount of at least a first binding ligand; wherein said binding ligand comprises a selected occluding agent operatively attached to a targeting agent and wherein said targeting agent binds to an aminophospholipid on the luminal surface of tumor vasculature.

3. (Amended) A method for destroying tumor vasculature, comprising administering to an animal having a vascularized tumor a tumor-destructive amount of at least a first binding ligand; wherein said binding ligand comprises a selected destructive agent operatively attached to a targeting agent and wherein said targeting agent binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

4. (Amended) A method for treating an animal having a vascularized tumor, comprising administering to said animal a therapeutically effective amount of at least a first binding ligand; wherein said binding ligand comprises at least a first therapeutic agent operatively attached to a targeting agent and wherein said targeting agent binds to an aminophospholipid on the luminal surface of blood vessels of a vascularized tumor.

5. The method of claim 4, wherein said targeting agent binds to phosphatidylethanolamine on the luminal surface of blood vessels of a vascularized tumor.

6. The method of claim 4, wherein said targeting agent binds to phosphatidylserine on the luminal surface of blood vessels of a vascularized tumor.

7. The method of claim 4, wherein said targeting agent comprises at least a first anti-aminophospholipid antibody or antigen-binding fragment thereof.

8. The method of claim 7, wherein said targeting agent comprises at least a first IgG or IgM anti-aminophospholipid antibody.

9. The method of claim 7, wherein said targeting agent comprises at least a first scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding region of an anti-aminophospholipid antibody.

10. The method of claim 7, wherein said targeting agent comprises at least a first human, humanized or part-human chimeric anti-aminophospholipid antibody or antigen-binding fragment thereof.

11. The method of claim 7, wherein said targeting agent comprises at least a first anti-aminophospholipid monoclonal antibody or antigen-binding fragment thereof.

12. The method of claim 11, wherein said targeting agent comprises at least a first anti-aminophospholipid monoclonal antibody, or antigen-binding fragment thereof, that is prepared by a preparative process comprising:

(a) preparing an anti-aminophospholipid antibody-producing cell; and

(b) fusing said anti-aminophospholipid antibody-producing cell with an immortal cell to prepare a hybridoma that produces said anti-aminophospholipid monoclonal antibody.

13. The method of claim 12, wherein said anti-aminophospholipid antibody-producing cell is obtained from a human patient having a disease associated with the production of anti-aminophospholipid antibodies.

14. The method of claim 12, wherein said anti-aminophospholipid antibody-producing cell is obtained by stimulating a mixed population of human peripheral blood lymphocytes with an immunogenically effective amount of an aminophospholipid sample.

15. The method of claim 12, wherein said anti-aminophospholipid antibody-producing cell is obtained by immunizing an animal with an immunogenically effective amount of an aminophospholipid sample.

16. The method of claim 15, wherein the immunized animal is a transgenic mouse that comprises a human antibody library and wherein the anti-aminophospholipid monoclonal antibody is a human monoclonal antibody.

17. The method of claim 12, wherein said preparative process comprises:

(a) obtaining anti-aminophospholipid antibody-encoding nucleic acids from said anti-aminophospholipid antibody-producing cell; and

(b) expressing said nucleic acids to obtain a recombinant anti-aminophospholipid monoclonal antibody.

18. The method of claim 12, wherein said preparative process comprises:

(a) immunizing an animal with an immunogenically effective amount of an aminophospholipid sample;

(b) preparing a combinatorial immunoglobulin phagemid library expressing RNA isolated from the spleen of the immunized animal;

(c) selecting from the phagemid library a clone that expresses an anti-aminophospholipid antibody; and

(d) expressing the anti-aminophospholipid antibody-encoding nucleic acids from said selected clone to provide a recombinant anti-aminophospholipid monoclonal antibody.

19. The method of claim 18, wherein the immunized animal is a transgenic mouse that comprises a human antibody library and wherein the recombinant anti-aminophospholipid monoclonal antibody is a recombinant human monoclonal antibody.

20. The method of claim 4, wherein said targeting agent comprises at least a first aminophospholipid binding protein or an aminophospholipid-binding fragment thereof.

21. The method of claim 20, wherein said targeting agent comprises at least a first annexin or a phosphatidylserine-binding fragment thereof.

22. The method of claim 21, wherein said targeting agent comprises at least a first Annexin V or a phosphatidylserine-binding fragment thereof.

23. The method of claim 20, wherein said targeting agent comprises at least a first phosphatidylethanolamine binding protein or a phosphatidylethanolamine-binding fragment thereof.

24. The method of claim 23, wherein said targeting agent comprises at least a first kininogen or a phosphatidylethanolamine-binding fragment thereof.

25. The method of claim 4, wherein said targeting agent comprises at least two aminophospholipid binding sites.

26. The method of claim 4, wherein said targeting agent is prepared by recombinant expression.

27. The method of claim 4, wherein at least two binding ligands are administered to said animal, wherein said binding ligands each bind to an aminophospholipid and comprise either distinct targeting agents or distinct therapeutic agents.

28. The method of claim 4, wherein said targeting agent is attached to at least a first anticellular or cytotoxic agent that kills or suppresses the growth or cell division of vascular endothelial cells.

29. The method of claim 28, wherein said targeting agent is attached to at least a first steroid, cytokine, antimetabolite, anthracycline, vinca alkaloid, antibiotic, alkylating agent, epipodophyllotoxin, DNA synthesis inhibitor, daunorubicin, doxorubicin or adriamycin.

30. The method of claim 28, wherein said targeting agent is attached to at least a first plant-, fungus- or bacteria-derived toxin.

31. The method of claim 30, wherein said targeting agent is attached to at least a first A chain toxin, bacterial endotoxin, lipid A moiety of bacterial endotoxin, ribosome inactivating protein, α-sarcin, gelonin, aspergillin, restrictocin, ribonuclease, diphtheria toxin or *Pseudomonas* exotoxin.

32. The method of claim 31, wherein said targeting agent is attached to at least a first ricin A chain or deglycosylated ricin A chain.

33. The method of claim 4, wherein said targeting agent is attached to at least a first coagulation factor.

34. The method of claim 33, wherein said targeting agent is attached to at least a first human coagulation factor.

35. The method of claim 33, wherein said targeting agent is attached to at least a first coagulation factor selected from the group consisting of Factor II/IIa, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, a vitamin K-dependent coagulation factor that lacks the Gla modification, Russell's viper venom Factor X activator, thromboxane A_2, thromboxane A_2 synthase and α2-antiplasmin.

36. The method of claim 33, wherein said targeting agent is attached to at least a first Tissue Factor, dimeric Tissue Factor, trimeric Tissue Factor, polymeric Tissue Factor, mutant Tissue Factor or Tissue Factor derivative.

37. The method of claim 36, wherein said targeting agent is attached to at least a first truncated Tissue Factor.

38. The method of claim 4, wherein said targeting agent is attached to at least two distinct therapeutic agents.

39. The method of claim 4, wherein said at least a first therapeutic agent is directly attached to said targeting agent by a direct covalent bond, via a chemical cross-linker or by recombinant expression as a fusion protein.

40. The method of claim 4, wherein said at least a first therapeutic agent is attached to said targeting agent via an antibody, or antigen binding region thereof, that binds to said therapeutic agent.

41. The method of claim 40, wherein said binding ligand is a bispecific antibody that comprises a first, targeting antibody, or antigen binding fragment thereof, that binds to an aminophospholipid; operatively attached to a second antibody, or antigen binding fragment thereof, that binds to said at least a first therapeutic agent.

42. The method of claim 4, wherein said binding ligand comprises an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

43. The method of claim 4, wherein said animal is a human patient.

44. The method of claim 4, wherein said at least a first therapeutic agent is operatively attached to said targeting agent using an avidin:biotin combination.

45. (Amended) A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first binding ligand effective to kill at least a portion of the intratumoral vascular endothelial cells; wherein said binding ligand comprises at least a first cytotoxic agent operatively attached to a targeting agent and wherein said targeting agent binds to an aminophospholipid expressed on the luminal surface of intratumoral blood vessels of the vascularized tumor.

46. A method for treating cancer, comprising administering to an animal having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first binding ligand effective to occlude or destroy intratumoral vasculature, the binding ligand comprising at least a first coagulative or destructive agent operatively attached to a targeting agent that binds to an aminophospholipid expressed on the luminal surface of intratumoral blood vessels of the vascularized tumor.

47. A method for treating cancer, comprising administering to an animal with a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first binding ligand effective to induce tumor necrosis, the binding ligand comprising at least a first therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid expressed on the luminal surface of intratumoral blood vessels of the vascularized tumor.

48. A method for treating an animal having a vascularized tumor, comprising administering to said animal a therapeutically effective amount of a pharmaceutical composition comprising at least a first construct comprising an anti-aminophospholipid antibody, or antigen binding fragment thereof, directly or indirectly linked to at least a first therapeutic agent.

49. The method of claim 48, wherein said construct comprises an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

50. (Amended) A method for delivering a selected therapeutic agent to tumor vasculature, comprising administering to an animal having a vascularized tumor a biologically effective amount of at least a first binding ligand; wherein said binding ligand comprises said selected therapeutic agent operatively attached to a targeting agent and wherein said targeting agent binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

EXHIBIT C
ALLOWED CLAIMS
APPLICATION SERIAL NO. 09/819,386

5. (Amended) A method for treating an animal having a vascularized tumor, comprising administering to said animal a therapeutically effective amount of at least a first binding ligand; wherein said binding ligand comprises at least a first therapeutic agent operatively attached to a targeting agent and wherein said targeting agent binds to an aminophospholipid on the luminal surface of blood vessels of a vascularized tumor; and further comprising:

(a) subjecting said animal to surgery or radiotherapy; or

(b) simultaneously or sequentially administering to said animal a therapeutically effective amount of at least a second anti-cancer agent.

6. The method of claim 5, wherein said targeting agent binds to phosphatidylethanolamine on the luminal surface of blood vessels of a vascularized tumor.

7. The method of claim 5, wherein said targeting agent binds to phosphatidylserine on the luminal surface of blood vessels of a vascularized tumor.

8. The method of claim 5, wherein said targeting agent comprises at least a first anti-aminophospholipid antibody or antigen-binding fragment thereof.

9. The method of claim 8, wherein said targeting agent comprises at least a first IgG or IgM anti-aminophospholipid antibody.

10. The method of claim 8, wherein said targeting agent comprises at least a first scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding region of an anti-aminophospholipid antibody.

11. The method of claim 8, wherein said targeting agent comprises at least a first human, humanized or part-human chimeric anti-aminophospholipid antibody or antigen-binding fragment thereof.

12. The method of claim 8, wherein said targeting agent comprises at least a first anti-aminophospholipid monoclonal antibody or antigen-binding fragment thereof.

21. The method of claim 5, wherein said targeting agent comprises at least a first aminophospholipid binding protein or an aminophospholipid-binding fragment thereof.

29. The method of claim 5, wherein said targeting agent is attached to at least a first anticellular or cytotoxic agent that kills or suppresses the growth or cell division of vascular endothelial cells.

30. (Amended) The method of claim 29, wherein said targeting agent is attached to at least a first steroid, cytokine, antimetabolite, anthracycline, vinca alkaloid, antibiotic, alkylating agent, epipodophyllotoxin, DNA synthesis inhibitor, daunorubicin, doxorubicin, adriamycin, A chain toxin, ricin A chain, deglycosylated ricin A chain, bacterial endotoxin, lipid A moiety of bacterial endotoxin, ribosome inactivating protein, α-sarcin, gelonin, aspergillin, restrictocin, ribonuclease, diphtheria toxin or *Pseudomonas* exotoxin.

34. The method of claim 5, wherein said targeting agent is attached to at least a first coagulation factor.

35. The method of claim 34, wherein said targeting agent is attached to at least a first human coagulation factor.

36. The method of claim 34, wherein said targeting agent is attached to at least a first coagulation factor selected from the group consisting of Factor II/IIa, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, a vitamin K-dependent coagulation factor that lacks the Gla modification, Russell's viper venom Factor X activator, thromboxane A_2, thromboxane A_2 synthase and α2-antiplasmin.

37. The method of claim 34, wherein said targeting agent is attached to at least a first Tissue Factor, dimeric Tissue Factor, trimeric Tissue Factor, polymeric Tissue Factor, mutant Tissue Factor or Tissue Factor derivative.

38. The method of claim 37, wherein said targeting agent is attached to at least a first truncated Tissue Factor.

46. The method of claim 5, further comprising subjecting said animal to surgery or radiotherapy.

47. The method of claim 5, further comprising simultaneously or sequentially administering to said animal a therapeutically effective amount of at least a second anti-cancer agent.

48. (Amended) The method of claim 47, wherein said at least a second anti-cancer agent is a chemotherapeutic agent, radiotherapeutic agent, cytokine, anti-angiogenic agent or apoptosis-inducing agent.

49. The method of claim 47, wherein said at least a second anti-cancer agent is an antibody-therapeutic agent construct comprising a second targeting antibody, or antigen binding fragment thereof, that binds to a surface-expressed, surface-accessible or surface-localized component of a tumor cell, tumor vasculature or tumor stroma; said targeting antibody or fragment thereof operatively linked to a therapeutic agent.

50. The method of claim 49, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to tumor cell or tumor stroma component.

51. The method of claim 49, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed, surface-accessible, surface-localized, cytokine-inducible or coagulant-inducible component of intratumoral blood vessels of a vascularized tumor.

52. The method of claim 51, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed component of intratumoral vasculature selected from the group consisting of an aminophospholipid, endoglin, a TGFβ receptor, E-selectin, P-selectin, VCAM-1, ICAM-1, PSMA, a VEGF/VPF receptor, an FGF receptor, a TIE, $\alpha_v\beta_3$ integrin, pleiotropin, endosialin and an MHC Class II protein.

53. The method of claim 51, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-localized component of intratumoral vasculature selected from the group consisting of VEGF/VPF, FGF, TGFβ, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor/hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF and TIMP.

54. The method of claim 49, wherein said second targeting antibody, or antigen-binding fragment thereof, is operatively linked to a cytotoxic agent.

55. The method of claim 49, wherein said second targeting antibody, or antigen-binding fragment thereof, is operatively linked to a coagulation factor or to an antibody, or antigen binding fragment thereof, that binds to a coagulation factor.

56. The method of claim 47, wherein said at least a second anti-cancer agent is a naked antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of intratumoral blood vessels of the vascularized tumor.

57. The method of claim 5, wherein said animal is a human patient.

65. A method for treating cancer, comprising simultaneously or sequentially administering to an animal having a vascularized tumor a therapeutically effective combination of a targeting agent-therapeutic agent construct comprising a therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second anti-cancer agent.

66. The method of claim 47, further comprising simultaneously administering to said animal a therapeutically effective amount of at least a second anti-cancer agent.

67. The method of claim 47, further comprising sequentially administering to said animal a therapeutically effective amount of at least a second anti-cancer agent.

68. The method of claim 67, wherein said at least a second anti-cancer agent is administered to said animal at a biologically effective time prior to said at least a first binding ligand.

69. The method of claim 68, wherein said at least a second anti-cancer agent injures or induces apoptosis in the endothelium of the blood vessels of said vascularized tumor.

70. (Amended) The method of claim 69, wherein said at least a second anti-cancer agent that injures or induces apoptosis in the endothelium of the blood vessels of said vascularized tumor is taxol, vincristine, vinblastine, neomycin, a combretastatin, a podophyllotoxin, TNF-α, angiostatin, endostatin, vasculostatin, an $\alpha_v\beta_3$ antagonist, a calcium-flux inducing agent or a calcium ionophore.

71. The method of claim 67, wherein said at least a second anti-cancer agent is administered to said animal at a biologically effective time subsequent to said at least a first binding ligand.

72. The method of claim 71, wherein said at least a second anti-cancer agent is an anti-tumor cell immunotoxin or an anti-angiogenic agent.

73. The method of claim 48, wherein said at least a second anti-cancer agent is a chemotherapeutic agent.

74. The method of claim 73, wherein said at least a second anti-cancer agent is a chemotherapeutic agent listed in Table C.

75. (Amended) The method of claim 73, wherein said at least a second anti-cancer agent is a chemotherapeutic agent selected from the group consisting of verapamil, cyclophosphamide, cytosine arabinoside, fluorouracil, methotrexate, aminopterin, mitomycin C, demecolcine, etoposide, mithramycin, chlorambucil, melphalan, daunorubicin, doxorubicin, tamoxifen, taxol, vincristine, vinblastine, camptothecin, actinomycin-D, cisplatin and a combretastatin.

76. The method of claim 48, wherein said at least a second anti-cancer agent is a radiotherapeutic agent.

77. The method of claim 48, wherein said at least a second anti-cancer agent is an anti-angiogenic agent.

78. The method of claim 77, wherein said at least a second anti-cancer agent is an anti-angiogenic agent listed in Table D.

79. The method of claim 78, wherein said at least a second anti-cancer agent is an anti-angiogenic agent selected from the group consisting of angiostatin, endostatin and angiopoietin.

80. The method of claim 48, wherein said at least a second anti-cancer agent is an apoptosis-inducing agent.

81. (New) The method of claim 21, wherein said targeting agent comprises at least a first annexin or a phosphatidylserine-binding fragment thereof.

82. (New) The method of claim 81, wherein said targeting agent comprises at least a first Annexin V or a phosphatidylserine-binding fragment thereof.

83. (New) The method of claim 81, wherein said targeting agent comprises Annexin V operatively attached to truncated Tissue Factor.

84. (New) The method of claim 47, wherein said at least a second anti-cancer agent is H_2O_2 or thrombin.

85. (New) The method of claim 47, wherein said at least a second anti-cancer agent is a compound that interferes with DNA replication, mitosis or chromosomal segregation.

86. (New) The method of claim 85, wherein said at least a second anti-cancer agent is taxol, vincristine, vinblastine, bleomycin, or a combretastatin.

87. (New) The method of claim 47, wherein said at least a second anti-cancer agent is a calcium flux inducing agent.

88. (New) The method of claim 87, wherein said at least a second anti-cancer agent is a calcium ionophore.

89. (New) The method of claim 48, wherein said at least a second anti-cancer agent is an inflammatory cytokine.

90. (New) The method of claim 89, wherein said at least a second anti-cancer agent is interleukin-4.

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-'kā-shən\ n (1697) 1 a : the natural process
: the hardening (as of muscular tissue) into a
mass or particle of ossified tissue 3 : a ten-
of being molded into a rigid, conventional, ster-
ndition
-,frāj\ n [L ossifraga, a bird of prey, fr. fem. of
ng, fr. oss-, os + frangere to break — more at
ERGEIER
ied; -fy-ing [L oss-, os + E -ify] vi (1713) 1 : to
: to become hardened or conventional and op-
t 1 : to change (as cartilage) into bone 2 : to
nal and opposed to change
o buc-co \,ō-sō-'bü-(,)kō, ,ó-sō-\ n [It ossobuco
l bone] (1935) : braised veal shanks
-ē, -syə-, -sə-\ n, pl -ar-ies [LL ossuarium, fr. L,
nes, fr. OL ossua, pl. of oss-, os] (1658) : a depos-
he dead
form [NL, fr. Gk, fr. osteon — more at OSSEOUS]
nyelitis⟩
j [ISV] (1877) : of, relating to, or resembling
or involving bone or the skeleton
\ n [NL] (ca. 1847) : inflammation of bone
t)-sə-bəl, ə-\ adj [F, fr. L ostensus, pp. of ostendere
in the way + tendere to stretch — more at OB-,
intended for display : open to view 2 : being
plausible rather than demonstrably true or real
e trip⟩ syn see APPARENT
dv (1765) 1 : in an ostensible manner 2 : to all

-siv\ adj (1782) 1 : OSTENSIBLE 2 2 : of, relating
finition by exemplifying the thing or quality being
e-ly adv
tən-'sōr-ē-əm, -,ten-, -'sór-\ n, pl -ria \-ē-ə\ [ML,
772) : MONSTRANCE
n-'tā-shən\ n [ME ostentacion, fr. MF ostentation,
entatio, fr. ostentare to display, freq. of ostendere]
display : PRETENTIOUSNESS 2 archaic : an act of

s\ adj (1673) : marked by or fond of conspicuous
sometimes pretentious display syn see SHOWY —
dv — os-ten-ta-tious-ness n
,äs-tē-ō-är-'thrī-təs\ n [NL] (1878) : arthritis
tion of the cartilage and bone of joints — os-te-o-
k\ adj
-ə-,blast\ n [ISV] (1875) : a bone-forming cell —
-tē-ə-'blas-tik\ adj
ə-,klast\ n [ISV oste- + Gk klastos broken — more
ny of the large multinucleate cells closely associated
esorption — os-te-o-clas-tic \,äs-tē-ə-'klas-tik\ adj
ə-,sīt\ n (1942) : a cell that is characteristic of adult
in a lacuna of the bone substance
s-tē-ə-'je-nə-səs\ n [NL] (1830) : development and

er-fec-ta \-,im-(,)pər-'fek-tə\ n [NL, imperfect os-
)1) : a hereditary disease marked esp. by extreme
ng bones
tē-ə-'je-nik\ adj (1867) 1 : producing bone 2
e
a n (ca. 1923) : OSTEOSARCOMA
d\ adj [ISV] (1840) : resembling bone
uncalcified bone matrix
ē-'ä-lə-jē\ n [NL osteologia, fr. Gk, description of
logia -logy] (1670) 1 : a branch of anatomy dealing
the bony structure of an organism — os-te-o-log-i-
\ adj — os-te-ol-o-gist \-tē-'ä-lə-jist\ n
'ō-mə\ n, pl -mas or -ma-ta \-mə-tə\ [NL] (ca.
mor composed of bone tissue
\,äs-tē-ō-mə-'lā-sh(ē-)ə\ n [NL, fr. oste- + Gk
fr. malakos soft — more at MOLLIFY] (ca. 1834) : a
hat is characterized by softening of the bones and is
ts in the immature
\-,mī-ə-'lī-təs\ n [NL] (1854) : an infectious inflam-
bone often of bacterial origin that is marked by local
on of tissue
ē-ə-,path\ n (1897) : a practitioner of osteopathy
-tē-'ä-pə-thē\ n [NL osteopathia, fr. oste- + L -pathia
system of medical practice based on a theory that
chiefly to loss of structural integrity which can be
pulation of the parts supplemented by therapeutic
of medicine or surgery) — os-te-o-path-ic \,äs-tē-ə-
s-te-o-path-i-cal-ly \-thi-k(ə-)lē\ adv
s-tē-ə-,plas-tē\ n (ca. 1860) : plastic surgery on bone;
of lost bone tissue or reconstruction of defective
te-o-plas-tic \,äs-tē-ə-'plas-tik\ adj
\,äs-tē-ō-pə-'rō-səs\ n, pl -ro-ses \-,sēz\ [NL, fr. oste-
tion, fr. porus pore + -osis] (1846) : a condition that
women and is characterized by decrease in bone mass

-ostosis *n comb form, pl* **-ostoses** *or* **-ostosises** [NL, fr. Gk ... *osteon* bone — more at OSSEOUS] : ossification of a (specified) part or to a (specified) degree ⟨hyper*ostosis*⟩

os·tra·cise *Brit var of* OSTRACIZE

os·tra·cism \'äs-trə-,si-zəm\ *n* (1588) **1** : a method of temporary banishment by popular vote without trial or special accusation practiced in ancient Greece **2** : exclusion by general consent from common privileges or social acceptance

os·tra·cize \-,sīz\ *vt* **-cized; -ciz·ing** [Gk *ostrakizein* to banish by voting with potsherds, fr. *ostrakon* shell, potsherd — more at OYSTER] (1649) **1** : to exile by ostracism **2** : to exclude from a group by common consent

os·tra·cod \'äs-trə-,käd\ *also* **os·tra·code** \-,kōd\ *n* [ultim. fr. Gk *ostrakon*] (1865) : any of a subclass (Ostracoda) of very small active mostly freshwater crustaceans that have the body enclosed in a bivalve carapace, the body segmentation obscured, the abdomen rudimentary, and only seven pairs of appendages

os·tra·co·derm \'äs-trə-kō-,dərm, äs-'tra-kə-\ *n* [ultim. fr. Gk *ostrakon* + *derma* skin — more at DERM-] (1891) : any of the early fossil jawless fishes of the Lower Paleozoic usu. having a bony covering of plates or scales

os·tra·con \'äs-trə-,kän\ *n, pl* **-tra·ca** \-trə-kə\ [Gk *ostrakon* potsherd, shell — more at OYSTER] (1883) : a fragment (as of pottery) containing an inscription — usu. used in pl.

os·trich \'äs-trich, 'ös- *also* -trij\ *n* [ME, fr. OF *ostrusce*, fr. (assumed) VL *avis struthio*, fr. L *avis* bird + LL *struthio* ostrich — more at STRUTHIOUS] (13c) **1 a** : a swift-footed 2-toed flightless ratite bird (*Struthio camelus*) of Africa that is the largest of existing birds and often weighs 300 pounds (140 kilograms) **b** : RHEA **c** : leather made from ostrich skin **2** [fr. the belief that the ostrich when pursued hides its head in the sand and believes itself to be unseen] : one who attempts to avoid danger or difficulty by refusing to face it — **os·trich-like** \-,līk\ *adj*

Os·tro·goth \'äs-trə-,gäth\ *n* [ME, fr. LL *Ostrogothi*, pl.] (14c) : a member of the eastern division of the Goths — **Os·tro·goth·ic** \,äs-trə-'gä-thik\ *adj*

Os·we·go tea \ä-'swē-gō-\ *n* [*Oswego* River, N. Y.] (1752) : a No. American mint (*Monarda didyma*) with showy scarlet irregular flowers

ot- *or* **oto-** *comb form* [Gk *ōt-*, *ōto-*, fr. *ōt-*, *ous* — more at EAR] : ear ⟨*ot*itis⟩ : ear and ⟨*oto*laryngology⟩

Othel·lo \ə-'the-(,)lō, ō-\ *n* : a Moor in the military service of Venice, husband of Desdemona, and protagonist of Shakespeare's tragedy *Othello*

¹oth·er \'ə-thər\ *adj* [ME, fr. OE *ōther*; akin to OHG *andar* other, Skt *antara*] (bef. 12c) **1 a** : being the one (as of two or more) remaining or not included ⟨held on with one hand and waved with the ~ one⟩ **b** : being the one or ones distinct from that or those first mentioned or implied ⟨taller than the ~ boys⟩ **c** : SECOND ⟨every ~ day⟩ **2** : not the same : DIFFERENT ⟨any ~ color would have been better⟩ ⟨something ~ than it seems to be⟩ **3** : ADDITIONAL ⟨sold in the U.S. and 14 ~ countries⟩ **4 a** : recently past ⟨the ~ evening⟩ **b** : FORMER ⟨in ~ times⟩

²other *n* (bef. 12c) **1 a** : one that remains of two or more **b** : a thing opposite to or excluded by something else ⟨went from one side to the ~⟩ **2** : a different or additional one ⟨the ~*s* came later⟩

³other *pron, sometimes pl in constr* (bef. 12c) **1** *obs* **a** : one of two that remains **b** : each preceding one **2** : a different or additional one ⟨something or ~⟩

⁴other *adv* (13c) : OTHERWISE — used with *than* ⟨was unable to see them ~ than by going to their home⟩

oth·er–di·rect·ed \,ə-thər-də-'rek-təd, -dī-\ *adj* (1950) : directed in thought and action primarily by external norms rather than by one's own scale of values — **oth·er–di·rect·ed·ness** *n*

oth·er·guess \'ə-thər-,ges\ *adj* [alter. of E dial. *othergates*] (1632) *archaic* : DIFFERENT

oth·er·ness \'ə-thər-nəs\ *n* (1587) **1** : the quality or state of being other or different **2** : something that is other or different

other than *prep* (1679) : with the exception of : EXCEPT FOR, BESIDES ⟨*other than* that, nothing happened⟩

oth·er·where \-,hwer, -,hwar, -,wer, -,war\ *adv* (14c) : ELSEWHERE

oth·er·while \-,hwīl, -,wīl\ *also* **oth·er·whiles** \-,hwīlz, -,wīlz\ *adv* (13c) *chiefly dial* : at another time

¹oth·er·wise \-,wīz\ *pron* [ME, fr. OE (*on*) *ōthre wīsan* in another manner] (bef. 12c) : something or anything else : something to the contrary ⟨do very little to enforce competition—and have never intended ~ —Milton Viorst⟩

²otherwise *adv* (13c) **1** : in a different way or manner ⟨glossed over or ~ handled —*Playboy*⟩ **2** : in different circumstances ⟨might ~ have left⟩ **3** : in other respects ⟨an ~ flimsy farce —*Current Biog.*⟩ **4** : if not ⟨do what I tell you, ~ you'll be sorry⟩ **5** : NOT — paired with an

P Q R

Phosphatidylserine is a marker of tumor vasculature and a potential target for anti-cancer drugs[1]

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Abbreviations used in this paper are: TF, tissue factor; VCAM-1, vascular cell adhesion molecule 1; IL-1a, interleukin-1 alpha; DPBS, Dulbecco's phosphate buffered saline; HRP; horseradish peroxidase; AP, alkaline phosphatase; SMC, smooth muscle cells; SCID, severe combined immunodeficient; PS, Phosphatidylserine; CL, cardiolipin; ROS, reactive oxygen species.

ABSTRACT

Phosphatidylserine (PS) is a phospholipid that almost exclusively resides on the inner leaflet of the plasma membrane under normal conditions. PS translocation to the external side of the membrane (PS exposure) is associated with apoptosis, necrosis, cell injury, cell activation and malignant transformation. We previously reported that endothelial cells in tumor vessels of Hodgkin's lymphoma implanted in mice express PS on their external surface. Endothelial cells in normal tissues did not express detectable amounts of PS. In the present study, we analyzed five additional tumor models for the exposure of PS on tumor vasculature and investigated a potential mechanism underlying this phenomenon. Anti-PS antibody specifically localized to tumor blood vessels in all tumors (HT29 human colon adenocarcinoma, NCI-H358 human lung carcinoma, B16 mouse melanoma, 3LL mouse lung carcinoma and Colo 26 mouse colon carcinoma). No localization was detected on normal endothelium. An isotype-matched control antibody directed against a different negatively charged lipid, cardiolipin, did not localize to either tumor or normal endothelium. Frozen tumor sections were examined for the presence of apoptotic cells, using a double labeling technique that detected a pan-endothelial cell marker and the apoptosis markers, active caspase 3 and fragmented DNA (Tunel assay). Neither apoptosis marker was present in tumor endothelium, indicating that PS-positive tumor vessels are seldom apoptotic. Externalization of PS also did not correlate with the maturation status of the vessels, as an abnormal pericytic network was equally evident around PS-positive and PS-negative tumor vessels. Various factors and tumor-associated conditions known to be present in the tumor microenvironment were examined for their ability to cause PS translocation in cultured endothelial cells. Hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines all induced PS exposure without causing cytotoxicity. Hydrogen peroxide was also a strong inducer. Possibly, conditions inside tumors generate reactive

oxygen species that induce PS exposure. Combined treatment with inflammatory cytokines and hypoxia/reoxygenation had greater than additive effects, suggesting that factors may interact to give amplified effects on PS-exposure on tumor endothelium *in vivo*. Since PS is absent from the outer surface of normal endothelium, its exposure on tumor vessels could potentially be utilized for tumor vessel targeting and imaging.

INTRODUCTION

Phosphatidylserine (PS) is a phospholipid that almost exclusively resides in the inner leaflet of the plasma membrane under normal conditions (1; 2). PS asymmetry is maintained by an ATP-dependent aminophospholipid translocase that is responsible for inward movement of aminophospholipids (3-5). Loss of PS asymmetry results from the outward movement of PS in the plasma membrane and is caused either by inhibition of the translocase (6; 7) or activation of scramblase, a Ca^{2+} dependent enzyme that transports lipids bidirectionally (8; 9). Loss of PS asymmetry is observed under different pathological and physiological conditions, including programmed cell death (10; 11), cell aging (12), intercellular fusion of myoblasts (13) and trophoblasts (14), cell migration (15; 16), activation of platelets (17-19) and cell degranulation (20). Endothelial cells externalize PS in response to thrombin (21) hyperlipidemia (22), viral infection (23), non-lytic concentrations of complement proteins C5b-9 (24) or exposure to calcium ionophore A23187 and PMA (25). Spontaneous PS exposure has been also observed in malignant cells in the absence of exogenous activators or cell injury (26-28).

Several major consequences follow membrane PS exposure. Phagocytic macrophages recognize, attach and eliminate PS-positive senescent and apoptotic cells (29; 30). PS also mediates attachment of T lymphocytes to thrombin-activated endothelial cells (21). The complement system is activated by PS and contributes to the lysis of PS-positive cells (31). Finally, PS exposure contributes to a procoagulant shift on the endothelium (1; 11) by providing a negatively charged lipid surface for assembly and activation of coagulation complexes (32; 33).

A mouse monoclonal IgM, 3SB, directed against PS has been raised and characterized by Rote and colleagues (17). The antibody binds to PS but not to phosphatidylcholine, phosphatidylethanolamine or cardiolipin (CL). 3SB binds to PS-coated ELISA plates in the presence or absence of serum, indicating that PS binding does not require cofactors. 3SB binds to cells having exposed PS (17). It is possible that it recognizes hexagonally packed PS, which has been reported to be antigenic (34).

We previously discovered that 3SB localized to tumor blood vessels in mice bearing human Hodgkin's disease tumors. This finding indicated that endothelial cells in Hodgkin's tumors, in contrast to those in normal tissues, expressed PS on the external surface of their plasma membrane. In the present study, we determined whether vascular PS exposure is observed in other types of tumors, and investigated the causes of PS translocation. PS exposure on tumor vasculature was present in all of six different tumors growing in mice. PS exposure was not due to apoptosis of tumor endothelium, or to irregularities in their coating with perivascular cells. Studies with cultured endothelial cells showed that hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines caused PS exposure without causing cytotoxicity. Hydrogen peroxide did likewise. Hypoxia/reoxygenation, acidity, thrombin and inflammatory cytokines may therefore act individually or collectively in tumors to generate peroxide ions and other reactive oxygen species that induce PS exposure on tumor endothelium.

PS on tumor vessels may provide a target molecule for the vascular targeting or imaging of vessels in solid tumors. Annexin V, an endogenous PS-binding ligand, has been used successfully to image PS-expressing activated platelets in thrombi (35), apoptotic cells in rejecting cardiac allografts, cyclophosphamide-treated lymphomas and anti-Fas antibody-

treated livers in rodents (10). Anti-PS antibodies may be directly cytotoxic to tumor vasculature, or mediate the binding of cytotoxic or coagulation factor to tumor vessels complement, host cells. Also, anti-PS antibodies, annexin V and other ligands that bind specifically to PS on tumor endothelial cells might be used to deliver a cytotoxic drug, radionuclide or coagulant to tumor vessels. Vascular targeting agents directed against markers on mature blood-transporting vessels in tumors have caused destruction of tumor vasculature and major tumor regressions in other systems (36-38). The present studies suggest the use of PS-directed antibodies and immunoconjugates for the vascular targeting or imaging of tumor vessels in man.

MATERIALS AND METHODS

Materials. $Na^{125}I$ was obtained from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle's tissue culture medium and Dulbecco PBS containing Ca^{2+} and Mg^{2+} were obtained from Gibco (Grand Usland, NY). Fetal calf serum was obtained from Hyclone (Logan, Utah). O-phenylenediamine, hydrogen peroxide and thrombin were from Sigma (St. Louis, MO). Flat bottom plates with 24 wells were obtained from Falcon (Becton Dickinson and Co., Lincoln Park, NJ). Recombinant murine interleukin-1 alpha, beta and tumor necrosis factor alpha (TNF α) were purchased from R&D Systems (Minneapolis, MN). Interferon of Universal Type I (hybrid protein that substitutes for all types of interferons) was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). Recombinant hepatocyte growth factor (HGF or scatter factor) and actinomycin D were from Calbiochem. Recombinant VEGF, PDGF-BB, $TGF\beta_1$, interleukin–6 (IL-6),

interleukin-8 (IL-8), interleukin-10 (IL-10) and FGF-1 were purchased from PeproTech (Rocky Hill, NJ).

Antibodies

Mouse monoclonal anti-PS and anti-CL IgM antibodies were raised as described (17). Both antibodies have been extensively characterized with regard to the specificity of their binding to PS and CL respectively (17). MECA 32, a pan mouse endothelial cell antibody, was kindly provided by Dr. E. Butcher (Stanford University, CA) and served as a positive control for immunohistochemical studies. Details of this antibody have been published (39). Rabbit anti-rat immunoglobulin, rat-anti mouse immunoglobulin and goat-anti mouse immunoglobulin secondary antibodies conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) were purchased either from Daco (Carpinteria, CA) or from Jackson Immunoresearch Labs (West Grove, PA).

Cells

L540Cy Hodgkin lymphoma cells, derived from a patient with end-stage disease, were provided by Prof. V. Diehl (Köln, Germany). HT29 human colon adenocarcinoma and NCI-H358 human non-small cell lung carcinoma were provided by Dr. Adi Gazdar (Southwestern Medical Center, Dallas, TX). B16 mouse melanoma and 3LL mouse lung carcinoma were obtained from American Type Cell Collection (Rockville, MD). Colo 26 mouse colorectal carcinoma was a gift from Dr. Ian Hart (ICRF, London, UK). The mouse brain endothelioma, bEnd.3, was provided by Prof. Werner Risau (Max Plank Institution, Munich, Germany).

METHODS

Tissue Culture

bEnd.3 cells, adult bovine aortic endothelial (ABAE) cells and all tumor cells except L540Cy lymphoma cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2 units/ml penicillin G and 2 ug/ml streptomycin. L540Cy cells were maintained in RPMI 1640 containing the same additives. Cells were sub-cultured once a week. Trypsinization of bEnd.3 cells was performed using 0.125% trypsin in PBS containing 0.2% EDTA. For *in vitro* studies, endothelial cells were seeded at a density of $1x10^4$ cells/ml in 1 ml of culture medium in 24 well plates and incubated 48-96 hours before being used in the assays. Medium was refreshed 24 hours before each experiment.

Growth of tumor cells *in vivo*

For localization studies, $2x10^7$ L540 human Hodgkin's lymphoma cells or $1x10^7$ cells of other tumor types were injected subcutaneously into the right flank of SCID mice (Charles River, Wilmington, MA). Tumors were allowed to reach a volume of 0.4-0.7 cm^3. A minimum of three animals per tumor group was used. Experiments were performed at least three times.

Detection of PS exposure on tumor endothelium *in vivo*

Detection of exposed PS *in vivo* was performed essentially as previously described (37). Briefly, anti-PS or anti-CL mouse IgM antibodies (30 ug/mouse) were injected intravenously in 200 ul of saline. Thirty minutes later mice were sacrificed and their blood circulation was exsanguinated and perfused with heparinized saline as previously described (40). All major organs and tumor were harvested and snap-frozen for preparation of

cryosections. Mouse IgM was detected using goat anti mouse IgM (μ specific) - HRP conjugate followed by development with carbazole (41). The number of positive vessels per high power field was determined at magnification of x 40. At least 10 random fields per section were examined and the average percentage of positive vessels was calculated.

Detection of apoptosis in tumors *in situ*

The blood circulation of mice was perfused with heparinized saline as previously described (40). Tumors were dissected out and snap-frozen for cryosectioning. Cytosolic and nuclear alterations characteristic for apoptotic cells were detected immunohistochemically by using two markers: active caspase-3 (42) and fragmented DNA (43). Active caspase-3 was detected by a rabbit anti-caspase-3 specific antibody (R&D, Minneapolis, MN) followed by incubation with anti-rabbit IgG conjugated to alkaline phosphatase (AP, Pierce, Rockford, IL). Other tumor sections were analyzed by Tunel assay (ApopTag kit, Oncor, MD) using anti-digoxigenin–alkaline phosphatase conjugate as a detecting reagent. To determine whether tumor endothelial cells express apoptotic markers, sections were sequentially labeled with MECA 32 (anti-endothelial marker) and anti rat-HRP secondary antibody, followed by either anti-caspase-3 antibody or Tunel assay and AP-conjugated secondary reagents. Vessels were visualized by their brown color, using Stable DAB (Research Genetics, Huntsville, AL) as a substrate. Apoptotic cells were identified by their pink-purple color (kit from Research Genetics) created by the phosphatase activity of secondary reagents detecting markers of apoptosis. The pink-purple stain was clearly distinguishable from the brown stain, if both markers coincided. These conditions of double labeling permitted the sequential detection of both enzymes.

Detection of pericytes on tumor frozen sections

To characterize interactions between PS-positive vessels and pericytes, tumor sections were double-labeled by goat anti mouse IgM-HRP (to identify anti-PS localized antibody) followed by anti α-smooth muscle cell actin (α-SMC) antibody (Daco, Carpinteria, CA), a marker of pericytes and vascular smooth muscle cells (SMC)(44; 45). The peroxidase activity was detected by Stable DAB and resulted in brown color. The pericytic marker was detected by anti mouse IgG-AP conjugate and resulted in a pink-purple color. Substrates for both enzymes were from Research Genetics. In other experiments, the endothelium was first detected by MECA 32 IgG and visualized by DAB, followed by anti α-SMC antibody and AP-mediated detection of pericytes/SMC.

Iodination of annexin V

Recombinant human annexin V was purified from E. coli transformed with ET12a-PAP1 plasmid (a gift from Dr. J. Tait, University of Washington, Seattle). The purity of the protein and the binding to PS were confirmed on SDS-PAGE and on PS-coated plastic, respectively. Rabbit polyclonal, affinity-purified anti-annexin V antibody was used to detect annexin V bound to PS. Annexin V was radiolabeled with ^{125}I using Chloramine T as described by Bocci (46). The specific activity was approximately 1×10^6 cpm per ug of protein, as measured by a Bradford assay (47).

Effect of growth factors, cytokines, inflammatory mediators, hydrogen peroxide, hypoxia and acidic pH on translocation of PS in cultured endothelial cells

Endothelial cells were treated with cytokines or growth factors at the concentrations listed in Table 3. All reagents were diluted in medium containing 10% serum and incubated with the cells at 37°C for 24 hours. To study the effect of hypoxia, cells were seeded on 24 well

plates and were incubated in a humidified normoxic atmosphere (21% O_2, 5% CO_2) for 48 hours before being transferred to a humidified hypoxic atmosphere (1% O_2, 5% CO_2, 94% N_2) in a sealed chamber (Billups Rothenberg inc, Del Mar, Ca). Cells were incubated in a hypoxic chamber for 24 hours at 37°C and the cells were compared to a parallel culture maintained under normoxic conditions. In some experiments, IL-1α (10 ng/ml) and TNFα (20 ng/ml) were added to medium prior to transfer to a hypoxic chamber.

To examine the effect of an acidic microenvironment, cells were exposed to the growth medium lacking bicarbonate, which was adjusted to different pHs (ranged between 7.3 to 6.2) with the required amount of HCl. Cells were incubated at 37°C in the absence of CO_2. Preservation of the medium pH in the presence of cell monolayer during 24 hours period was confirmed in each experiment. Under these experimental conditions all tested reagents were non-toxic to either bovine or mouse endothelial cells and had no effect on cell morphology or viability of the attached monolayer.

Detection of exposed PS on cultured endothelial cells by ^{125}I-labeled annexin V

After treatment with the reagents described above, treated and control cells were incubated with 7.1 pmoles of ^{125}I-labeled annexin V (200 ul/well) in the binding buffer. After 2 hours incubation at room temperature, cells were washed extensively and dissolved in 0.5 M of NaOH. The entire volume of 0.5 ml was transferred to plastic tubes and counted in a gamma counter. Non-specific binding was determined in the presence of 5 mM EDTA and was subtracted from experimental values. The results were expressed as net pmoles of cell-bound annexin V, normalized per 1×10^6 cells. Maximal binding of annexin V was determined on cells simultaneously treated with actinomycin D and TNFα (50 ng/ml of each component). As has been previously reported, combination of the above agents causes apoptosis and PS exposure in the 100 percent of the treated endothelial cells (48).

Basal binding of ^{125}I-annexin V to untreated cells was determined in the presence of medium with 10% serum. The amount of ^{125}I-annexin V that bound to the untreated cultures was subtracted from that in the treated cultures. The specific increase in the amount of externalized PS was calculated according to the following formula: (net experimental binding / net maximal binding) X 100. Each experiment was performed in duplicate and was performed at least three times.

Detection of exposed PS on endothelial cells *in vitro* by biotinylated annexin V

Endothelial monolayers were washed with DPBS containing Ca^{2+} and Mg^{2+} and fixed with 0.25% of glutaraldehyde diluted in the same buffer. Excess aldehyde groups was quenched by incubation with 50 mM of Na_4Cl for 5 minutes. Cells were washed with DPBS (containing Ca^{2+}, Mg^{2+} and 0.2% gelatin) and incubated with 1 ug/ml of biotinylated annexin V (Pharmingen, San Diego, CA). After 2 hours of incubation, cells were washed with 0.2% gelatin buffer incubated with streptavidin-HRP (1:500 dilution). Detection of PS *in situ* was confirmed by staining with anti-PS IgM. Anti-CL IgM and streptavidin were used as negative controls in these assays. All steps were performed at room temperature. PS-positive cells were detected by addition of carbazole substrate, resulting in insoluble red-brownish precipitate. The number of positive cells per high power field was determined and expressed as a percent of the total number of cells. Six random fields were scored per well and the average was calculated.

RESULTS

PS is a marker of tumor vasculature.

PS exposure on vascular endothelium *in vivo* was detected by a specific anti-PS mouse IgM, (3SB) as previously described (37). A class and species matched antibody, D11 (17),

directed against a different negatively charged phospholipid, CL, served as a negative control. CL exclusively resides in the membrane of mitochondria (49). This anti-CL monoclonal antibody recognizes CL, not PS or other lipids (17).

All six tumors included in this study contained PS-positive vessels (Fig.1 and Table 1). The percentage of PS-positive vessels ranged from 40% in B16 tumors to 10% in Colo 26 tumors. Anti-PS IgM was present on the luminal surface of capillaries and venules in all regions of the tumors. PS-positive vessels appeared to be particularly prevalent in and around regions of necrosis. Positive vessels usually did not show morphological abnormalities that were apparent by light microscopy. Occasional vessels located in necrotic areas showed morphological signs of deterioration. Detection of PS by 3SB was specific since no staining of tumor endothelium was observed with the anti-CL antibody. Anti-PS antibody (but not anti-CL antibody) also localized to necrotic and apoptotic tumor cells. No vascular localization of anti-PS or anti-CL antibodies was observed in normal organs other than the kidneys (Table 1). In the kidneys, tubules were stained in both anti-PS and anti-CL recipients, presumably because of secretion of IgM through this organ. These findings demonstrate that PS is present on the luminal surface of vascular endothelial in various tumors but not in normal tissues.

PS-positive tumor vessels are not apoptotic

A double labeling technique was used to identify apoptotic endothelial cells in tumor sections. Apoptotic cells were identified with two independent markers: an active form of caspase-3, which identifies cytosolic changes in dying cells (42), and fragmented DNA, which identifies cells having nuclear alterations (43). Active caspase-3 was detected by a specific antibody. Fragmented DNA was visualized by Tunel assay (43). Cells positive for apoptotic markers were stained pink by AP-labeled secondary reagents. Endothelial cells in blood vessels were stained brown by HRP-labeled by a pan-endothelial antibody, MECA

32. Both colors were clearly visible on the same cells, if endothelial and apoptotic markers coincided.

Endothelial cells in five out of six types of tumors (HT29, H358, B16, Colo 26, L540) did not display either of the apoptosis markers (Fig. 2, Table 2). The sixth type of tumor, 3LL, displayed a few apoptotic endothelial cells that were located in necrotic areas. In contrast, apoptotic malignant cells were common in all types of tumors. The percentage of apoptotic tumor cells ranged from 1-2% in L540 tumors to 12.6-19.6% in 3LL tumors. There was broad correspondence between the number and location of tumor cells that stained positively for active caspase 3 and for fragmented DNA. However, cells displaying active caspase 3 were about 1.5 times as abundant as those with fragmented DNA, probably because active caspase 3 is an earlier and less specific marker of apoptosis than is fragmented DNA.

Lack of correlation between PS-positive tumor vessels and abnormal distribution of pericytes

We explored whether abnormalities in the perivascular cell coating of tumor vessels might account for their PS exposure by determining whether there was a correlation between the integrity of the coating by pericytes and the positivity for externalized PS. Frozen tumor sections from mice that had been injected with anti-PS antibody or anti-CL antibody were double labeled for the presence of the vascular endothelial cell marker, MECA 32, and a pericytic cell/SMC marker, αSMC-actin. Endothelial cells were stained brown while pericytic cells were stained pink. PS-positive tumor vessels were identified in sequential sections by staining for localized anti-PS IgM (brown) followed by identification of pericytes (pink). In contrast to the continuous pericytic layer surrounding vascular

endothelial cells in normal tissues, vessels in all tumors had a discontinuous and disorganized network of pericytic cells (Fig. 3). Vessels in B16 melanomas and Colo 26 carcinomas almost completely lacked pericytes. HT29 and H358 carcinomas contained pericytes/SMC that were often totally separated from endothelial cells. The L540 lymphoma was the only tumor of those examined that had a major percentage (about 40%) of vessels in which pericytic cells were properly attached.

There was no correlation between irregularities in pericytic cells and exposure of PS on tumor vessels (Fig. 3). PS-positive and PS-negative tumor endothelium had equal proportions of coated vessels, uncoated vessels and vessels with partly detached pericytic layers. Abnormalities in pericyte coating, therefore, do not appear to be the cause of PS translocation on tumor endothelium.

Inducers of PS translocation by endothelial cells *in vitro*

Endothelial cells *in vitro* were treated with non-toxic concentrations of various factors and conditions that are present in the microenvironment of many tumors (50-54). The factors included: angiogenic factors (VEGF, HGF and bFGF) tumor and host cell–derived inflammatory and pro-angiogenic cytokines (IL-1α, IL-1β, TNFα, IL-6, IL-8, IL-10 and interferons), thrombin, hypoxia/reoxygenation , oxygen-reactive species and low pH. These conditions are all known to perturb endothelial cells and could be considered as potential cause of PS translocation. Activation, perturbation and injury of endothelium by these factors and conditions are associated with a rise of intracellular Ca^{2+} concentration. The rise in intracellular Ca^{2+} might activate scramblase (9) and simultaneously inhibit aminophospholipid translocase (6), leading to accumulation of PS on the external side of the membrane.

Mouse bEnd.3 or bovine ABAE cells were treated for 24 hours with different concentrations of potential inducers of PS translocation. PS externalization was quantified by measuring ^{125}I-annexin V binding. The amount of annexin V binding was compared with that of cells in which apoptosis of 100% of cells had been induced by combined treatment with actinomycin D and TNF-α. Actinomycin D and TNF-α induced the binding of 6.2 pmoles of annexin V per 10^6 cells (3.8 x 10^6 molecules of annexin V per cell) on both cell types, in good agreement with literature reports (55). This value was taken as the maximal amount of externalized PS.

Untreated cells were largely devoid of externalized PS, as judged by annexin V or anti-PS antibody binding (Table 3, and Fig. 4A). The basal binding in the presence of growth medium alone was 0.44 and 0.68 pmoles of ^{125}I-annexin V for ABAE and bEnd.3 cells, respectively. This corresponds to 7.06% and 10.9% of the maximal binding for ABAE and bEnd.3 cells, respectively, which correlated well with the finding that approximately 10% of cells bound biotinylated annexin V under the same conditions. VEGF, HGF, FGF, TGFβ_1, PDGF, IL-6, IL-8 and IL-10 did not increase binding of ^{125}I-annexin V above the basal level for untreated cells. Inflammatory mediators (IL-1α, IL-1β, TNFα and interferon) caused a small but reproducible increase in PS translocation that ranged from 6 to 8% of the maximal level for ABAE cells and from 7 to 14% for bEnd3 cells (Table 3). Hypoxia/reoxygenation, thrombin or acidic external conditions (pH 6.8-6.6) induced a moderately high externalization of PS that ranged from 8 to 20% of the maximal level for ABAE cells and from 17 to 22% of the maximal level for bend.3 cells. The largest increase in PS translocation was observed after treatment with 100 to 200 uM of hydrogen peroxide. This treatment caused nearly complete (95%) externalization of PS in both cell types as judged by ^{125} I-annexin V binding (Table 3). More than 70% of ABAE and bEnd.3

cells bound biotinylated annexin V, as judged immunohistochemically (Fig. 4B). PS-expressing endothelial cells generated by treatment with hypoxia/reoxygenation, thrombin, acidity, TNFα, IL-1 or H_2O_2 remained attached to the matrix during time period of the assay (24 hours), retained cell-cell contact (Fig. 4B) and retained their ability to exclude trypan blue dye. Normal PS orientation was restored 24 to 48 hours later in the majority of the cells. These results indicate that mild oxidative stress, created by direct application of H_2O_2 or indirectly by hypoxia/reoxygenation, acidity, thrombin, or inflammatory cytokines, triggers a transient translocation of PS on viable endothelial cells.

Combined Effects of Inflammatory Cytokines and Hypoxia/Reoxygenation on PS-Exposure by Endothelial Cells *In Vitro*

Enhanced PS exposure was observed when ABAE cells were subjected to hypoxia/reoxygenation in the presence of IL-1α or TNFα. In the absence of the cytokines, hypoxia/reoxygenation increased PS-exposure by ABAE cells to 15% of the maximum level for cells treated with apoptotic concentrations of actinomycin D and TNFα. In the presence of subtoxic concentrations of IL-1α or TNFα, hypoxia/reoxygenation increased PS-exposure to 26% and 33% respectively of the maximum (Fig. 5). Cytokines in the absence of hypoxia/reoxygenation increased PS-exposure by less than 7% indicating that the combination of cytokines and hypoxia/reoxygenation had greater than additive effects on PS-exposure. Thus, in tumors, the PS-inducing effect of hypoxia/reoxygenation may be amplified by inflammatory cytokines and possibly by such other stimuli as acidity and thrombin.

DISCUSSION

The major finding to emerge from this study is that vascular endothelial cells in tumors externalize PS to their luminal surface where it can be bound by a specific anti-PS antibody *in vivo*. PS is absent from the external surface of vascular endothelial cells in normal tissues, suggesting that PS-recognizing antibodies, annexin V and other ligands might be used for delivering cytotoxic drugs, coagulants or radionuclides for the selective destruction or imaging of vessels in solid tumors.

PS-positive tumor endothelium appears to be viable. It does not display markers of apoptosis, it is morphologically intact and metabolically active, as indicated by its expression of VCAM-1, E-selectin and other rapidly turned-over proteins. Although often regarded as an indicator of apoptosis, PS exposure has been observed in several types of viable cells, including malignant cells (27; 28; 55), activated platelets (17), and embryonic trophoblasts at various stages of migration, matrix invasion and fusion (14). Recent studies suggest that constitutive PS exposure on malignant cells may occur because expression of truncated (less active) aminophospholipid translocase, as detected by epitope-specific antibodies against the putative PS translocase, ATPaseII[4]. Lack of correlation between PS exposure and commitment to cell death has been also shown on pre-apoptotic B lymphoma cells that restored PS asymmetry and grew normally after removal of pro-apoptotic stimulus (56). In normal viable cells, PS exposure is probably triggered by surface events, such as ligand-receptor interactions, that induce Ca^{2+} fluxes into the cells (57). Ca^{2+} fluxes activate scramblase (9) and simultaneously inhibit aminophospholipid translocase (6).

To shed light on the mechanism of PS exposure on tumor endothelial cells, a series of experiments was performed in which endothelial cells *in vitro* were treated with various factors and conditions known to be present in the tumor microenvironment. The objective was to identify factors that induce PS exposure on endothelial cells without causing cytotoxicity in order to mimic the situation in tumors *in vivo*. Hypoxia followed by reoxygenation, acidity, and thrombin increased PS exposure on viable endothelial cells to between 10 and 22% of the level seen when all cells are apoptotic. Inflammatory cytokines (TNFα and IL-1α) also caused a weak but definite induction of PS exposure. The possibility that these conditions are in fact the major inducing stimuli in tumors *in vivo* is suggested by the following: i) PS positive endothelium is prevalent in and around regions of necrosis where hypoxia, acidity, thrombosed blood vessels, and infiltrating host leukocytes are commonly observed; ii) the finding that hypoxia/reoxygenation amplifies the weak PS-exposing activity of TNFα and IL-1 on endothelial cells *in vitro* (Fig. 5) correlates with the situation *in vivo* in tumors where hypoxia and cytokine-secreting tumor and host cells co-exist; iii) hypoxia/reoxygenation and thrombin have been reported to generate reactive oxygen species (e.g. peroxides) in endothelial cells through activation of NADPH oxidase-like membrane enzyme (58; 59). Hydrogen peroxide was the most powerful inducer of PS exposure on cultured endothelial cells found in the present study, providing indirect support for the involvement of reactive oxygen species. Based on these findings we propose the following cascade of events leading to PS exposure on tumor vessels, which indirectly contributes to tumor-associated thrombosis (Fig. 6). Hypoxia/reoxygenation in combination with inflammatory cytokines, thrombin and acidity are responsible in part for generation of ROS by endothelial and tumor cells. ROS are also produced by infiltrating host cells (macrophages, neutrophils and granulocytes) that are

attracted by necrosis and tumor-derived cytokines. Tumor endothelial cells respond to rising ROS concentration by transferring PS to the external side of the membrane. The externalized PS provides the negative phospholipid surface upon which coagulation factors concentrate and assemble. This confers the procoagulant status to the tumor endothelium that has long been recognized. PS also provides an attachment site for circulating macrophages (29), T lymphocytes (21) and polymorphonuclear cells that assist in leukocyte infiltration into tumors. Adherence of activated macrophages, polymorphonuclear cells and platelets to PS on tumor endothelium may lead to further secretion of reactive oxygen species and further amplification of PS exposure.

Anti-PS antibodies might be used for cancer therapy in several ways. Unconjugated antibodies might directly suppress tumor endothelial cell growth or survival by interfering with critical PS-dependent surface functions. This hypothesis is supported by the studies on viable B cells demonstrating that PS neutralization inhibits signaling from the cell surface via B cell receptor (57). Anti-PS antibodies might also mediate toxicity by binding complement and cytotoxic cells, by inducing apoptosis or by promoting pro-thrombotic status of tumor vascular endothelium. In certain autoimmune disorders, anti-aminophospholipid antibodies cause normal tissue damage by analogous mechanisms (60-62). Also, anti-PS antibodies or ligands might be linked to various effector molecules (e.g. cytotoxic drugs, radionuclides, coagulants) to create vascular targeting agents that destroy or occlude blood vessels in solid tumors. Such agents have been shown to be highly effective, and sometimes curative, in mice with large solid tumors (36; 38).

PS on tumor vessels is attractive as a target for several reasons: it is abundant (minimum of 3 x 10^6 molecules per cell); it is on the luminal surface of tumor endothelium, which is

directly accessible for binding by vascular targeting agents in the blood; it is present on a high percentage of tumor endothelial cells in diverse solid tumors, and it is absent from endothelium in all normal tissues examined to date. Unconjugated antibodies, vascular targeting agents and imaging agents directed against PS on tumor vasculature potentially could have utility for cancer treatment in man.

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Table 1 Specific localization of anti-PS and anti-CL antibodies to tumor vessels mice

Tissue	Anti-PS[a]	Anti-cardiolipin
L540 tumor	++[b]	-
H358 tumor	++	-
HT29 tumor	+	-
B16 tumor	+++	-
3LL tumor	++	-
Colo 26 tumor	++	-
Adrenal	-	-
Brain	-	-
Heart	-	-
Kidney	-[c]	-[c]
Intestine	-	-
Liver	-	-
Lung	-	-
Pancreas	-	-
Spleen	-	-
Testis	-	-

[a] Localization of anti-PS or anti-CL antibody in tumor bearing mice was determined by injecting the antibody, perfusing the blood circulation of the mice with saline and detecting the antibody on sections of the tissues by using an anti-mouse IgM - peroxidase conjugate.

[b] Intensity of staining was compared to pan-endothelial marker MECA 32; - indicates no staining; + weak (fewer than 5% positive vessels) ; ++ moderate (5 to 20 %); +++ (20 to 40 %) strong.

[c] Non-antigen specific tubular staining was visible in both anti-PS and anti-CL recipients.

Table 2 **Expression of apoptotic markers in tumors**

Tumor type	Active caspase-3		Tunel assay	
	Tumor cells (% of total)[a]	Tumor vessels	Tumor cells (% of total)	Tumor vessels
3LL	19.8	<1.0[b]	12.6	0
HT29	13.7	0	7.8	0
H358	5.8	0	4.3	0
Colo 26	5.3	0	4.1	0
B16	4.2	0	3.5	0
L540	2.3	0	1.6	0

[a] The percentage of tumor cells or tumor blood vessels that were positive for either caspase-3 or Tunel was determined in ten high power fields per section. The fields were randomly selected along two perpendicular directions from the edges through the center of the tumor. The average of the percentage of positive cells or vessels in tumor from 3 mice is presented.

[b] Occasional vessels (1 of >100) in the necrotic area of 3LL tumor displayed both markers of apoptosis.

Table 3 Effect of cytokines, growth factors and stress conditions on exposure of PS on endothelial cells *in vitro*.

Treatment	**Concentration**[a]	**^{125}I-Annexin V (% of Max binding)** [b]	
		ABAE cells	bEnd.3 cells
Medium with 10% serum	N/A [c]	0	0
Actinomycin D + TNF α	50 ng/ml each	100	100
VEGF	20 ng/ml	0	0
βFGF	20 ng/ml	0	0
Scatter factor	40 ng/ml	0	0
TGF β_1	20 ng/ml	0	0
PDGF-BB	20 ng/ml	0	0
IL-10	20 ng/ml	0	0
IL-8	20 ng/ml	0	0
IL-6	20 ng/ml	0	0
IL-1α	10 ng/ml	6.4	7.5
IL-1β	10 ng/ml	5.8	5.5
Interferon	40 ng/ml	8.6	2.8
TNFα	20 ng/ml	7.4	13.7
Thrombin	50 nM	8.8	17.4
Hypoxia	1% O_2	15.0	22.5
pH 6.6 [d]	N/A	20.2	18.9
Hydrogen peroxide	100 uM [e]	95.5	98.4

[a] Concentrations of cytokines, growth factors and thrombin were selected from literature values to have maximal stimulatory effect on cultured endothelial cells. These concentrations did not cause toxicity over the period of the test (24 hours) as judged by morphological appearance, a lack of detachment, and a lack of uptake of trypan blue.

[b] Binding of ^{125}I-annexin V was performed as described under Methods. Basal binding was determined in the presence of growth medium alone. Maximal PS exposure was determined after induction of apoptosis by the combined treatment with actinomycin D and TNF α. Untreated ABAE and bEnd.3 cells bound 0.44 and 0.68 pmoles of ^{125}I-Annexin V, respectively. Maximal binding was 6.2 pmoles of ^{125}I-annexin V for both cell types (equivalent to 3.8×10^6 molecules per cell) The percentage of increase of annexin V binding was calculated according to the following formula: (net experimental binding / net maximal binding) X 100. Average of duplicates from three separate experiments is presented. SE was less than 5%.

[c] Not applicable.

[d] Cells were exposed to the growth medium lacking bicarbonate that had been adjusted to pH 6.6 with 1N HCl. Cells were incubated at 37^{0}C in the absence of CO_2.

[e] The maximal concentration of H_2O_2 that did not cause cytotoxicity under chosen conditions.

Figure Legends:

Fig. 1. Localization of anti-PS antibody to vascular endothelial cells in L540 human Hodgkin's lymphoma, 3LL murine lung carcinoma and B16 murine melanoma tumors in mice. Tumor-bearing SCID mice were injected intravenously with 20 ug of anti-PS or anti-CL mouse IgM. The blood circulation was perfused with saline one hour later. Mice were sacrificed one hour later and tumor and organs were harvested and snap-frozen. Mouse IgM was detected on frozen sections using goat anti-mouse IgM-peroxidase conjugate. Anti-PS antibody specifically localized to blood vessels (indicated by arrows) in all tumors. No localization was observed in mice injected with control anti-CL IgM.

Fig. 2. Lack of apoptotic vascular endothelial cells in 3LL and L540 tumors. Frozen sections of 3LL and L540 tumors were double labeled with a pan-endothelial cell antibody, MECA 32 to visualize blood vessels, and with anti-apoptotic markers detecting either active caspase-3 or fragmented DNA (Tunel assay). MECA 32 staining of vessels is indicated by the brown color (arrows). Expression of apoptotic markers is indicated by the pink color (arrowheads). Both apoptotic markers are present in some tumor cells but are absent from tumor endothelium.

Fig 3. Pericyte-endothelial cell interactions in PS-positive and PS-negative vessels in 3LL tumors. Mice bearing 3LL tumors were injected with 20 ug of anti-PS IgM. PS-positive vessels (*upper panel*) were detected as described above. PS-negative vessels (defined as MECA 32-positive, anti-PS-negative) were identified on serial sections of the same tumor (*lower panel*). Vessels were labeled brown with the pan-endothelial cell marker. Pericytes were identified with anti-αSMC actin antibody and were stained pink (arrowheads). Representative fields of PS-positive, pericyte-negative (*upper left*), PS-positive, pericyte-positive (*upper right*), PS-negative, pericyte-negative (*lower left*) and PS-negative, pericyte-positive (*lower right*) vessels are shown. Note the detachment of the pericytic layer from tumor endothelium and the uneven distribution of pericytes along tumor vessels (*upper and lower right*).

Fig. 4. Externalized PS on bEnd.3 mouse endothelial cells treated with 100 uM of hydrogen peroxide. A) untreated bEnd.3 cells; B) bEnd.3 cells after treatment with 100 uM of hydrogen peroxide. PS is absent from the surface of untreated cells but becomes externalized after treating the cells with H_2O_2. PS-positive cells remain attached to the substratum, retain cell-cell contact and other morphological signs of viability.

Fig. 5. Synergistic effect of hypoxia and inflammatory cytokines on PS exposure. bEnd.3 cells were treated for 24 hours with IL-1 α and TNF α under normoxic and hypoxic conditions. PS externalization was determined on viable endothelial monolayer by measuring binding of ^{125}I-annexin V. The increase in PS exposure was calculated as explained under "Materials and Methods".

Fig. 6. Hypothesis for induction of PS exposure on tumor vessels and its contribution to the procoagulant shift of the tumor endothelium.

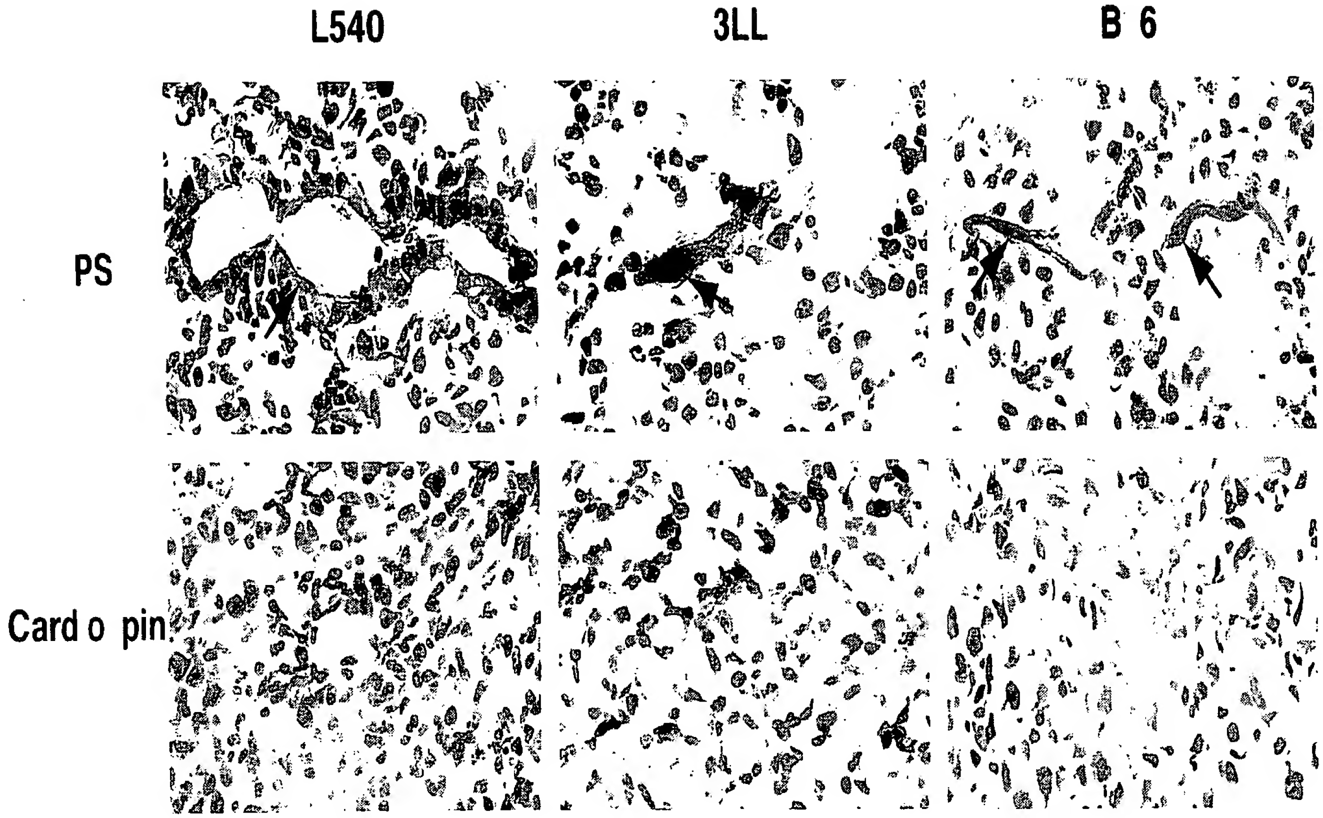

FIG. 1

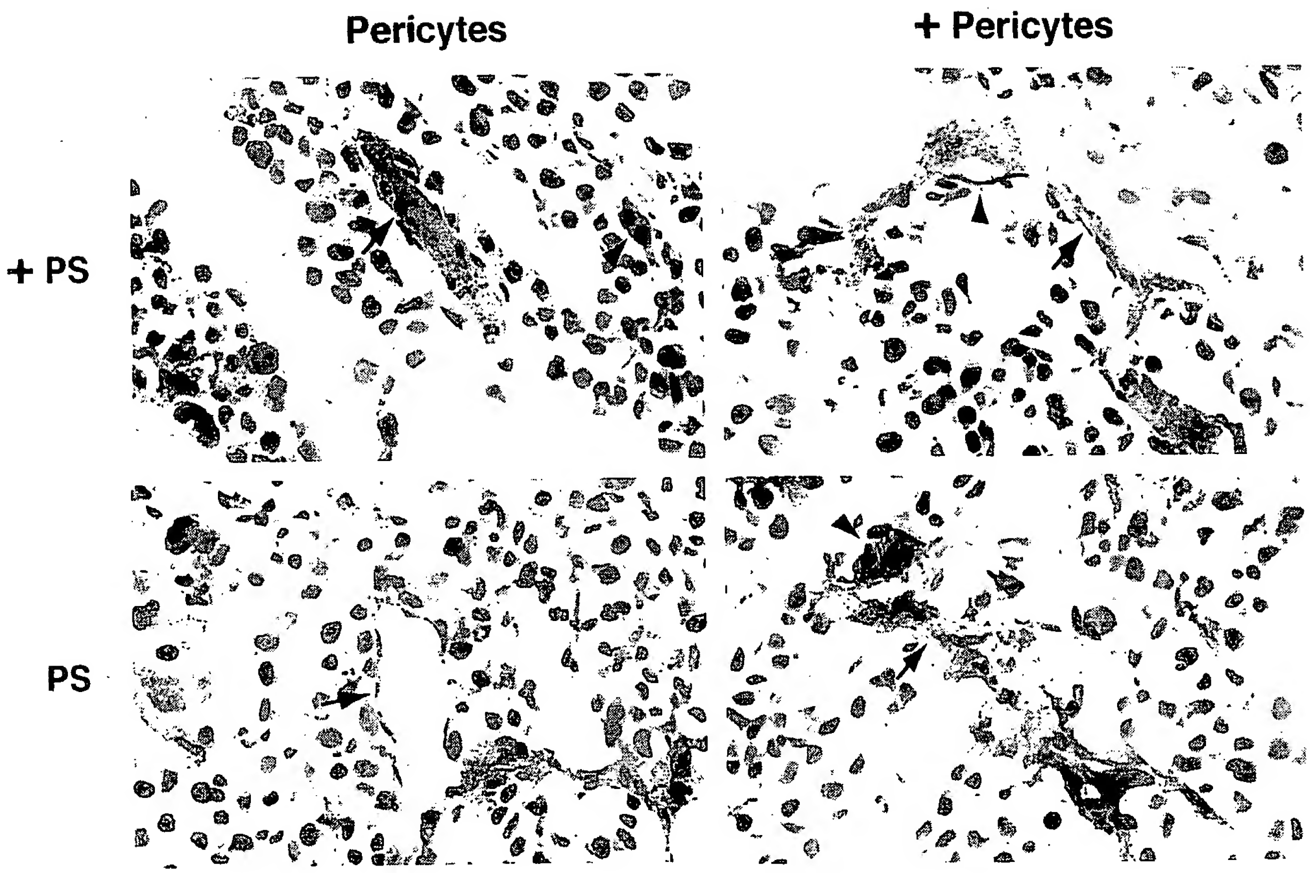

FIG. 2

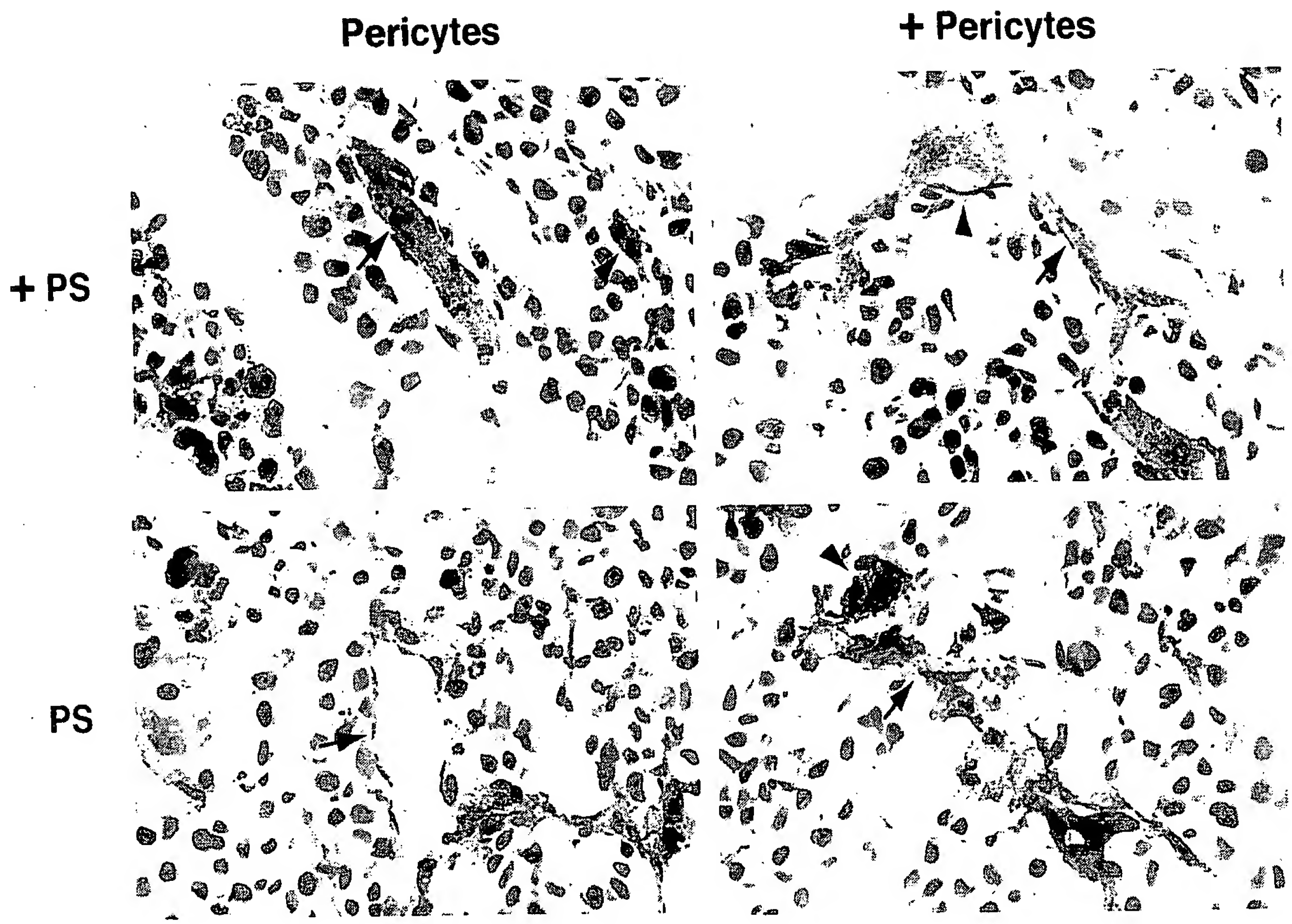

FIG. 3

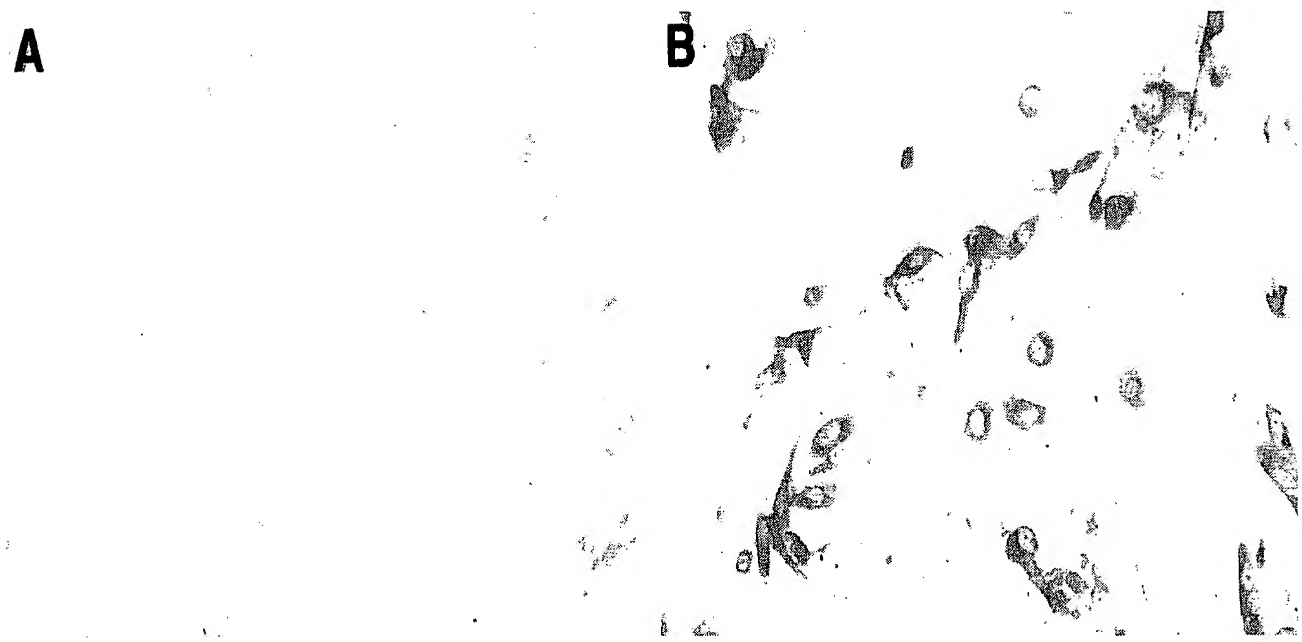

FIG. 4

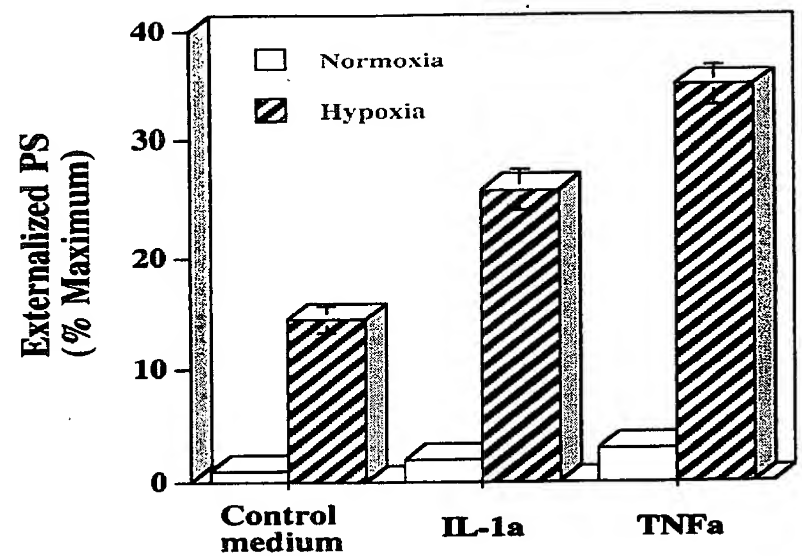
Externalized PS
(% Maximum)
40
30
20
10
0
Normoxia
Hypoxia
Control
medium
IL-1a
TNFa

FIG. 5

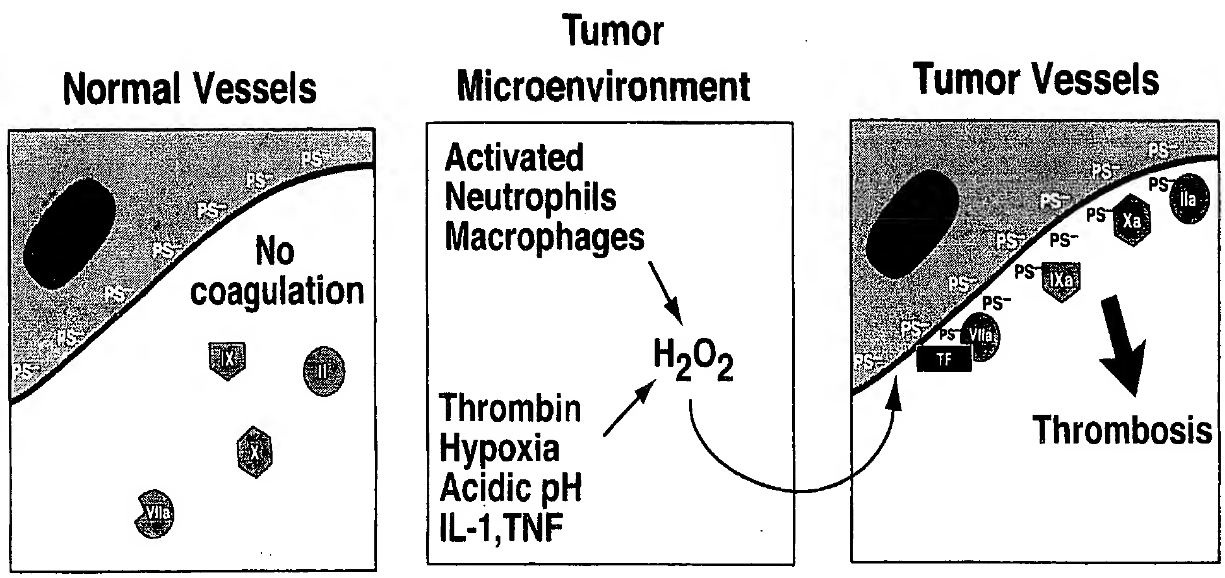
Normal Vessels
PS
No
coagulation
IX
II
X
VIIa
Tumor
Microenvironment
Activated
Neutrophils
Macrophages
H_2O_2
Thrombin
Hypoxia
Acidic pH
IL-1,TNF
Tumor Vessels
PS
IIa
Xa
IXa
VIIa
TF
Thrombosis

FIG. 6

[CANCER RESEARCH 62, 6132–6140, November 1, 2002]

Increased Exposure of Anionic Phospholipids on the Surface of Tumor Blood Vessels[1]

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ABSTRACT

Anionic phospholipids are largely absent from the external leaflet of the plasma membrane of mammalian cells under normal conditions. Exposure of phosphatidylserine on the cell surface occurs during apoptosis, necrosis, cell injury, cell activation, and malignant transformation. In the present study, we determined whether anionic phospholipids become exposed on tumor vasculature. A monoclonal antibody, 9D2, which specifically recognizes anionic phospholipids, was injected into mice bearing a variety of orthotopic or ectopic tumors. Other mice received annexin V, a natural ligand that binds to anionic phospholipids. Both 9D2 and annexin V specifically localized to vascular endothelium in all of the tumors, and also to tumor cells in and around regions of necrosis. Between 15 and 40% of endothelial cells in tumor vessels were stained. No localization was detected on normal endothelium. Various factors and tumor-associated conditions known to be present in the tumor microenvironment were examined for their ability to cause exposure of anionic phospholipids in cultured endothelial cells, as judged by 9D2 and annexin V binding. Hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines all induced exposure of anionic phospholipids. Hydrogen peroxide was also a strong inducer. Combined treatment with inflammatory cytokines and hypoxia/reoxygenation had greater than additive effects. Possibly, injury and activation of tumor endothelium by cytokines and reactive oxygen species induce exposure of anionic phospholipids, most likely phosphatidylserine. Anionic phospholipids on tumor vessels could potentially provide markers for tumor vessel targeting and imaging.

INTRODUCTION

Anionic phospholipids are largely absent from the surface of resting mammalian cells under normal conditions. PS,[3] which is the most abundant anionic phospholipid of the plasma membrane, is tightly segregated to the internal leaflet of the plasma membrane in most cell types (1, 2). PI, another major anionic phospholipid, is also situated predominantly in the internal leaflet of the plasma membrane (3). The minor anionic phospholipids, PA and PG, have only been examined in a few cells types, but they also appear to be mainly situated in the internal leaflet of the plasma membrane (4). CL, another anionic phospholipid, is present in the mitochondrial membrane and is absent from the plasma membrane (5). The neutral phospholipids are also asymmetrically distributed in the plasma membrane; PE is predominately on the internal leaflet, whereas the choline-containing phospholipids, PC and SM, are predominantly on the external leaflet.

PS asymmetry, along with that of PE, is maintained by an ATP-dependent aminophospholipid translocase that catalyzes the transport of aminophospholipids from the external leaflet to the internal leaflet of the plasma membrane (6). Loss of PS and PE asymmetry results from the outward movement of these phospholipids in the plasma membrane and is caused either by inhibition of the translocase (7) or activation of scramblase, a Ca^{2+}-dependent enzyme that transports all of the lipids bidirectionally (8). Loss of asymmetry is observed under different pathological and physiological conditions, including apoptosis (9), cell activation (10, 11), injury (12), and malignant transformation (13). Exposure of PS also plays a role in intercellular fusion (14, 15) and cell migration (16). Endothelial cells externalize PS in response to increased Ca^{2+} fluxes induced by thrombin (17), calcium ionophore or phorbol esters (18), hyperlipidemia (19) and nonlytic concentrations of complement proteins C5b-9 (20).

Several major consequences follow membrane PS exposure. Phagocytic macrophages recognize, attach, and eliminate PS-positive senescent and apoptotic cells (21, 22). PS also mediates attachment of T lymphocytes to thrombin-activated endothelial cells (17). The complement system is activated by PS and contributes to the lysis of PS-positive cells (23). Finally, PS exposure contributes to a procoagulant shift on the endothelium (1, 9) by providing a negatively charged lipid surface for assembly and activation of coagulation complexes (24, 25). The prothrombotic character of the tumor endothelium has long been recognized (26).

In the present study, we hypothesized that anionic phospholipids become exposed on tumor vasculature because of increased stress conditions of the tumor microenvironment. Injury and activation of tumor endothelium have been shown to be caused by: (*a*) tumor-derived interleukin-1 and tumor necrosis factor, which activate the endothelium and induce expression of cell adhesion molecules (27, 28); (*b*) ROS generated by leukocytes that adhere to the endothelium (28); and (*c*) ROS generated by tumor cells themselves as a byproduct of metabolism (27, 29) or as a result of exposure to hypoxia followed by reoxygenation (30). These observations suggested that Ca^{2+} fluxes might be generated by these stresses within the tumor endothelium that, in turn, cause exposure of PS, and probably also of PE, through activation of scramblase or inhibition of aminophospholipid translocase.

To detect cell surface anionic phospholipids, we generated a new monoclonal antibody, 9D2, which reacts with anionic but not neutral phospholipids. 9D2 antibody is more specific for anionic phospholipids than is the natural ligand, annexin V, which strongly binds to PE, in addition to anionic phospholipids. Annexin V has been used successfully to image activated platelets in thrombi, apoptotic cells in cardiac allografts undergoing rejection, cyclophosphamide-treated lymphomas, and anti-Fas antibody-treated livers in rodents (31). We found that 9D2 and annexin V localize specifically to tumor endothelium after i.v. injection to mice bearing various types of solid tumors. This finding indicates that anionic phospholipids, most likely PS, routinely become exposed on the surface of tumor vascular endothelium.

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[3] The abbreviations used are: PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; ROS, reactive oxygen species; IL, interleukin; TNF, tumor necrosis factor; DPBS, Dulbecco's phosphate buffered saline; HRP; horseradish peroxidase; SCID, severe combined immunodeficient; VEGF, vascular endothelial growth factor; ABAE, adult bovine aortic endothelial; DAB, 3,3′-diaminobenzidine.

MATERIALS AND METHODS

Materials. $Na^{125}I$ was obtained from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle's tissue culture medium and Dulbecco PBS containing Ca^{2+} and Mg^{2+} were obtained from Life Technologies, Inc. (Grand Island, NY). FCS was obtained from Hyclone (Logan, UT). L-α-PS, L-α-PC, CL, L-α-PE, L-α-PI, SM, PA, PG, *O*-phenylenediamine, hydrogen peroxide, and thrombin were from Sigma (St. Louis, MO). Flat-bottomed plates with 24 wells were obtained from Falcon (Becton Dickinson and Co., Lincoln Park, NJ). Recombinant hepatocyte growth factor (or scatter factor) and actinomycin D was from Calbiochem (San Diego, CA). Recombinant murine interleukin-1 α, β, and TNF-α were purchased from R&D Systems (Minneapolis, MN). IFN of universal type I (hybrid protein that substitutes for all types of IFNs) was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). Recombinant human VEGF 121, human platelet-derived growth factor-BB, IL-6, IL-8, IL-10, and human fibroblast growth factor-2 were purchased from PeproTech (Rocky Hill, NJ).

Antibodies. MECA 32, a pan mouse endothelial cell antibody, was kindly provided by Dr. Eugene Butcher (Stanford University, Stanford, CA) and served as a positive control for immunohistochemical studies. Details of this antibody have been published (32). Rabbit antirat immunoglobulin, rat-antimouse immunoglobulin, and goat-antimouse and antirat secondary antibodies conjugated to HRP were purchased either from DAKO (Carpinteria, CA) or from Jackson Immunoresearch Labs (West Grove, PA).

Cells. L540Cy Hodgkin lymphoma cells, derived from a patient with end-stage disease, were provided by Prof. Volker Diehl (Medizinische Universitätsklinik 1, Köln, Germany). NCI-H358 human non-small cell lung carcinoma was provided by Dr. Adi Gazdar (Southwestern Medical Center, Dallas, TX). Meth A mouse fibrosarcoma and MDA-MB-231 human breast carcinoma were obtained from American Type Cell Collection (Rockville, MD). The mouse brain endothelioma line, bEnd.3, was provided by Prof. Werner Risau (Max Plank Institution, Munich, Germany). ABAE cells were purchased from Clonetics (San Diego, CA).

Tissue Culture. bEnd.3, ABAE cells, and all of the tumor cells except L540Cy lymphoma were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 2 units/ml penicillin G, and 2 μg/ml streptomycin. L540Cy cells were maintained in RPMI 1640 containing the same additives. Cells were subcultured once a week. Trypsinization of bEnd.3 cells was performed using 0.125% trypsin in PBS containing 0.2% EDTA. For *in vitro* studies, endothelial cells were seeded at a density of 1×10^4 cells/ml in 1 ml of culture medium in 24-well plates and incubated 48–96 h before being used in the assays. Medium was refreshed 24 h before each experiment.

Growth of s.c. Implanted Tumors. For localization studies, 2×10^7 L540 human Hodgkin's lymphoma cells or 1×10^7 cells of other tumor types were injected s.c. into the right flank of SCID mice (Charles River, Wilmington, MA). Tumors were allowed to reach a volume of 0.4–0.7 cm^3. A minimum of three animals per group was used. Experiments were replicated at least three times.

Orthotopic Model of Human MDA-MB-231 Breast Carcinoma. Female *nu/nu* or SCID mice were purchased from Charles River. MDA-MB-231 human mammary carcinoma cells were implanted into the mammary fat pad according to a published protocol (33). Briefly, mice were anesthetized, and a 5-mm incision was made in the skin over the lateral thorax. The mammary pad was exposed to ensure the correct site for injection of 1×10^7 MDA-MB-231 cells resuspended in 0.1 ml of saline.

Generation of 9D2 Rat Monoclonal Antibody Reactive with Anionic Phospholipids. To generate monoclonal antibodies reactive with anionic phospholipids, female Lewis rats were immunized with bEnd.3 endothelial cells that had been treated with 200 μM of hydrogen peroxide for 2 h. The treatment caused translocation of anionic phospholipids to the external surface in 70–90% of cells as detected by ^{125}I-labeled annexin V. Treated cells were washed, detached, and counted. Two-million cells were suspended in sterile PBS and injected five times i.p. with the interval of 3 weeks between injections. The titer of polyclonal antibodies to anionic phospholipids was determined 2 days after each immunization. Hybridomas were obtained by fusing splenocytes from immunized rats with myeloma partner P3 × 63AG8.653 cells (American Type Culture Collection). The reactivity of the selected antibody, rat IgM 9D2, with PS and CL was established by screening hybridoma supernatants on PS, CL, PE, and PC immobilized on plastic. Additional characterization of phospholipid specificity of 9D2 is given in "Results."

Reactivity of 9D2 Antibody and Annexin V with Plastic-immobilized Phospholipids. Phospholipids were dissolved in *n*-hexane to a concentration of 50 μg/ml. One-hundred μl of this solution was added to wells of 96-well microtiter plates. After evaporation of the solvent in air, the plates were blocked for 2 h with 10% fetal bovine serum diluted in DPBS containing 2 mM Ca^{2+} (binding buffer). 9D2 antibody or annexin V were diluted in the binding buffer in the presence of 10% serum at an initial concentration of 6.7 nM. Serial 2-fold dilutions were prepared in the plates (100 μl per well). The plates were then incubated for 2 h at room temperature. The plates were washed, and the 9D2 and annexin V were detected by goat antirat IgM conjugated to HRP and rabbit antihuman annexin V followed by goat antirabbit IgG conjugated to HRP (all diluted 1:1000), respectively. Secondary reagents were detected by using chromogenic substrate O-phenylenediamine followed by reading plates at 490 nm using a microplate reader (Molecular Devices, Palo Alto, CA). The specificity of the 9D2 antibody binding was validated by using control rat IgM of irrelevant specificity (PharMingen, San Diego, CA). The specificity of annexin V binding to phospholipids, which is Ca^{2+}-dependent, was determined by diluting the reagent in the DPBS containing 5 mM EDTA. Additional negative controls consisted of washing the plates with the binding buffer containing 0.2% of a detergent Tween 20. This treatment extracts lipids, thus removing the phospholipid that was absorbed to plastic. Neither 9D2 antibody nor annexin V bound to detergent-washed plates.

Detection of Externally Positioned Anionic Phospholipids by 9D2 Antibody and Annexin V on the Surface of Cultured Endothelial Cells. Endothelial cells were grown until they reached ~70% confluence. To induce PS exposure, cells were treated with H_2O_2 (200 μM) for 1 h at 37°C. Control and treated slides were washed with DPBS containing Ca^{2+} and Mg^{2+}, and fixed with 0.25% of glutaraldehyde diluted in the same buffer. Excess aldehyde groups were quenched by incubation with 50 mM of NH_4Cl for 5 min. To examine the effect of detergents and organic solvents on detection of phospholipids, some slides were preincubated with acetone (5 min) or with PBS containing 1% (v/v) Triton X-100. Cells were washed with DPBS containing Ca^{2+}, Mg^{2+}, and 0.2% (w/v) gelatin, and incubated with 1 μg/ml of biotinylated annexin V (PharMingen) or with 1 μg/ml of 9D2 antibody. After 2 h of incubation, cells were washed with 0.2% gelatin buffer and were incubated with streptavidin-HRP (1:500 dilution) or with antirat-HRP. Rat IgM of irrelevant specificity and streptavidin alone were used as negative controls in these experiments. All of the steps were performed at room temperature. HRP activity was measured by adding *O*-phenylenediamine (0.5 mg/ml) and hydrogen peroxide (0.03% w/v) in citrate-phosphate buffer (pH 5.5). After 15 min, 100 μl of supernatant were transferred to 96-well plates, 100 μl of 0.18 M H_2SO_4 were added, and the absorbance was measured at 490 nm. Alternatively, PS-positive cells were detected by addition of carbazole substrate, resulting in insoluble red-brownish precipitate. Each experiment was performed in duplicate and repeated at least twice.

Inhibition of 9D2 and Annexin V Binding to Phospholipids by Liposomes. Specificity of phospholipid recognition was additionally confirmed by competition assays with various liposomes. Liposomes were prepared from solutions of 5 mg of a single phospholipid in chloroform. The solutions were dried under nitrogen to form a thin layer in a round-bottomed glass flask. Ten ml of Tris buffer (0.1 M; pH 7.4) were then added, and the flask was sonicated five times for 2 min. 9D2 or annexin V (6.66 nM) were preincubated with 200 μg/ml of liposomal solution for 1 h at room temperature. The mixture was added to phospholipid-coated plates or endothelial cell monolayers. The ability of 9D2 to bind to an immobilized phospholipid or cell surface in the presence or absence of the different liposomes was determined as described above.

Competition of 9D2 and Annexin V for Binding to Immobilized PS. Biotinylated 9D2 antibody and annexin V were prepared by incubating purified proteins with a 10-fold molar excess of *N*-hydroxysuccinimide biotin (Sigma) for 1 h at room temperature. Free biotin was removed by dialysis against PBS. The biotinylation procedure did not impair the PS-binding capacity of either protein. For competition experiments, unmodified and biotinylated proteins were premixed with a 10-fold molar excess of unmodified proteins. The mixtures were then added to PS-coated plates. Bound reagents were detected by streptavidin-HRP conjugate diluted 1:1000. The binding to PS of each reagent in the absence of a competitor was taken as the 100% value.

Detection of Externally Positioned Anionic Phospholipids by 9D2 Antibody and Annexin V in Tumor-bearing Mice *in Vivo*. Immunohistochemical techniques, in which 9D2 or annexin V are applied directly to sections of frozen tissues, do not discriminate between anionic phospholipids on the inner leaflet and the outer leaflet of the plasma membrane. To detect externally positioned phospholipids, 50 μg of 9D2 or biotinylated 9D2 antibody, or 100 μg of biotinylated annexin V were injected i.v. into tumor-bearing SCID mice. Sixty min later mice were sacrificed, and their blood circulation was exsanguinated and perfused with heparinized saline as described previously (34). All of the major organs and tumor were removed and snap-frozen for preparation of cryosections. Sections were blocked with PBS containing 10% serum. To prevent loss of phospholipids during slide processing, detergents and organic solvents were omitted from blocking and washing buffers. Rat IgM was detected using goat antirat IgM (μ-specific) HRP conjugate followed by development with carbazole or DAB (35). This procedure was successfully used to detect PS-positive vessels in L540Cy tumors by using the mouse anti-PS antibody 3SB (36). Biotinylated reagents were detected by streptavidin conjugated to HRP. Tumor sections derived from mice injected with saline or rat IgM of irrelevant specificity served as negative controls. Additional controls consisted of incubating the slides in 1% Triton solution or in acetone for 10 min. These treatments extract phospholipids. No signal was detected under these conditions. Staining of the sections by this method for the presence of 9D2 or annexin V detects cells having externalized anionic phospholipids that were accessible for binding by the reagents *in vivo*.

Identification and Quantification of PS-positive Tumor Vessels. Structures with localized 9D2 antibody or annexin V were identified as blood vessels by morphological appearance on DAB-stained sections and by coincident staining with the pan-endothelial cell marker, MECA 32, on serial sections of frozen tissues. Quantification on DAB-stained sections was done by counting vessels stained by MECA 32, 9D2, or annexin V in serial sections of a tumor. Six slides of each tumor type derived from 6 mice injected with 9D2 antibody, control rat IgM, or annexin V were examined. At least 10 random fields/section (0.317 mm^2/field) were scored in blinded fashion by two independent observers. The mean numbers and SEs of vessels stained by 9D2, annexin V, or MECA 32 were calculated. The mean number of 9D2 or annexin V-positive vessels determined in each tumor type group was compared with the mean number of MECA 32-positive vessels in the same tumor group. The percentage of 9D2 or annexin V-positive vessels was calculated.

In additional experiments, mice bearing MDA-MB-231 tumors (0.3–0.7 cm^3 in volume) were i.v. injected with 50 μg of biotinylated 9D2, control IgM, or annexin V (6 mice per group). Biotinylated reagents were first incubated with streptavidin-Cy3 conjugate, washed in PBS, then incubated with MECA 32 antibody followed by FITC-tagged antirat IgG secondary antibody. Single images, taken with appropriate filters for Cy3 (red) and FITC (green) fluorescence, respectively, were captured by digital camera and transferred to a computer. Images of 10 random fields (0.317 mm^2/field) demonstrating yellow color (a product of merged green and red fluorescence) were superimposed with the aid of Metaview software. The same method was used to analyze tumors from mice injected with control rat IgM or saline. The percentage of vessels with localized 9D2 or annexin V was calculated as follows: mean number of yellow vessels per field divided by mean number of green (total) vessels multiplied by 100.

Iodination of Annexin V. Recombinant human annexin V was purified from *Escherichia coli* transformed with ET12a-panionic phospholipid1 plasmid (a gift from Dr. Jonathan Tait, University of Washington, Seattle, WA). The purity of the protein and the binding to PS were confirmed on SDS-PAGE and on PS-coated plastic, respectively. Rabbit polyclonal, affinity-purified antiannexin V antibodies were used to detect annexin V bound to PS. Annexin V was radiolabeled with ^{125}I using chloramine T as described by Bocci (37). The specific activity was $\sim 1 \times 10^6$ cpm/μg of protein, as measured by a Bradford assay (38).

Detection of Exposed PS on Cultured Endothelial Cells by ^{125}I-labeled Annexin V. After treatment with the reagents described above, treated and control cells were incubated with 7.1 pmols of ^{125}I-labeled annexin V (200 μl/well) in the binding buffer. After 2 h incubation at room temperature, cells were washed extensively and dissolved in 0.5 M of NaOH. The entire volume of 0.5 ml was transferred to plastic tubes and counted in a gamma counter. Nonspecific binding was determined in the presence of 5 mM EDTA and was subtracted from experimental values. The results were expressed as net pmols of cell-bound annexin V, normalized per 1×10^6 cells. Maximal binding was determined on cells simultaneously treated with actinomycin D and TNF-α (50 ng/ml of each component). As has been reported previously, the above agents cause apoptosis and PS exposure in 90–100% of endothelial cells (39). Basal binding of ^{125}I-annexin V to untreated cells was determined in the presence of medium with 10% serum. The amount of ^{125}I-annexin V that bound to the untreated cultures was subtracted from that in the treated cultures. The specific increase in the amount of externalized PS was calculated according to the following formula: (net experimental binding/net maximal binding) × 100. Each experiment was performed in duplicate and was performed at least three times.

Effect of Growth Factors, Cytokines, Inflammatory Mediators, Hydrogen Peroxide, Hypoxia, and Exposure to Low pH on Exposure of PS in Cultured Endothelial Cells. Endothelial cells were treated with cytokines or growth factors at concentrations listed in Table 3. All of the reagents were diluted in medium containing 10% serum and incubated with the cells at 37°C for 24 h. To study the effect of hypoxia, cells were seeded on 24-well plates and were incubated in a humidified normoxic atmosphere (21% O_2, 5% CO_2) for 48 h before being transferred to a humidified hypoxic atmosphere (1% O_2, 5% CO_2, 94% N_2) in a sealed chamber (Billups Rothenberg Inc., Del Mar, CA). Cells were incubated in a hypoxic chamber for 24 h at 37°C and were then returned to a normoxic environment for 4 h at 37°C. The cells were compared with a parallel culture from an identical passage, seeded on the same day, and maintained entirely under normoxic conditions. To examine the effect of an acidic microenvironment, cells were exposed to the growth medium lacking bicarbonate, which was adjusted to different pHs (ranging between 7.3 and 6.2) with HCl. Cells were incubated at 37°C in the absence of CO_2. It was confirmed that culture medium held the assigned pH during the 24-h period of culture. These experimental conditions were not toxic to either bovine or

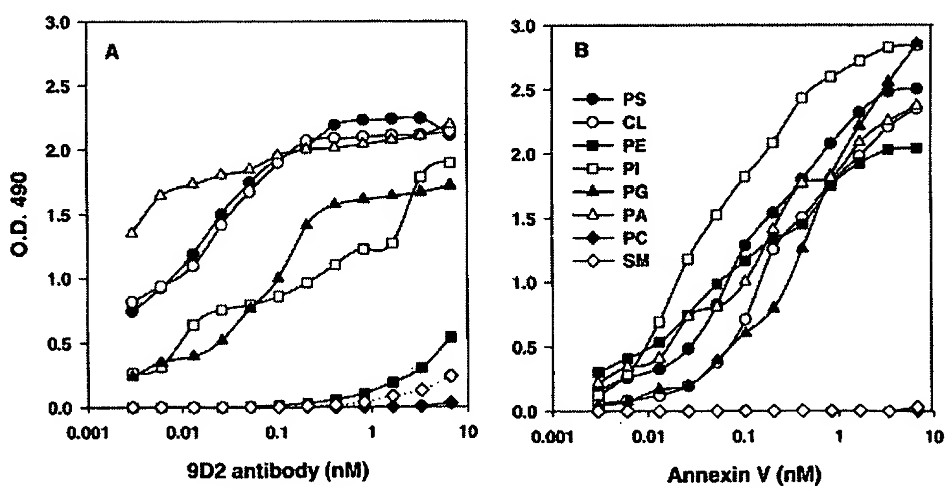

Fig. 1. Binding of 9D2 antibody and annexin V to phospholipids adsorbed to plastic. Phospholipids were adsorbed to plastic of microtiter plates (see "Materials and Methods"). After blocking with 10% serum, 9D2 antibody or annexin V were added at concentrations ranging from 6.7 nM to 0.005 nM in the presence of 10% serum. The plates were washed, and the bound 9D2 antibody and annexin V were detected using goat antirat IgM-HRP and rabbit antiannexin V IgG followed by antirabbit-HRP, respectively.

mouse endothelial cells, and had no effect on cell morphology or viability of the attached monolayer.

RESULTS

Phospholipid Specificity of 9D2 Antibody and Annexin V. 9D2 antibody specifically recognized anionic phospholipids (PS, PA, CL, PI, and PG) and had no significant reactivity with neutral phospholipids (PE, PC, and SM) in ELISA (Fig. 1; Table 1). The order of strength of binding of 9D2 to phospholipids in ELISA was PA>PS = CL>PG = PI. The binding was antigen-specific because no binding was observed with several control rat IgM of irrelevant specificity. Also, binding of 9D2 to any of the anionic phospholipids adsorbed to ELISA plates was blocked by liposomes prepared from any of the anionic phospholipids but not by liposomes prepared from any of the neutral phospholipids. Annexin V also bound to anionic phospholipids but its binding was less specific than that of 9D2, in that it also bound strongly to the neutral phospholipid, PE. The order of strength of binding of annexin V to phospholipids in ELISA was PI>PS = PE = PA = CL>PG (Table 1). These findings for annexin V are consistent with earlier data (40). Neither 9D2 nor annexin V bound detectably to heparin, heparan sulfate, or to double- or single-stranded DNA (data not shown). The binding of 9D2 was unaffected by the presence of 5 mM EDTA, showing it did not require Ca^{2+} for binding to anionic phospholipids. In contrast, the binding of annexin V to anionic phospholipids was abolished in the presence of 5 mM EDTA, as expected from its known dependence on Ca^{2+} for binding to anionic phospholipids or PE (41, 42). Neither 9D2 nor annexin V bound to ELISA plates that had been coated with phospholipids but then washed with 0.2% Tween in saline, confirming that their binding was to the absorbed phospholipids.

9D2 Antibody and Annexin V Do Not Cross-Block Each Other's Binding to PS. To examine whether 9D2 antibody and annexin V compete for binding to PS, cross-blocking experiments were performed using biotinylated proteins on PS-coated plates. Binding of biotinylated 9D2 antibody and annexin V was blocked by a 10-fold molar excess of unmodified 9D2 and annexin V, respectively (Table 2). However, unmodified annexin V did not affect the ability of biotinylated 9D2 to bind to the PS plate. Likewise, addition of unmodified 9D2 antibody did not alter the ability of biotinylated annexin V to bind to the PS plate (Table 2). These results indicate that 9D2 antibody and annexin V do not cross-block each other binding to PS-coated plates, either because they recognize different epitopes on the PS molecule or different conformations of PS adsorbed on plastic.

Table 1 *Phospholipid specificity of 9D2 antibody and annexin V*

Phospholipid		Abundance and location in the plasma membrane under normal conditions[a]	EC_{50} of binding (pM)	
Name	Type		9D2	Annexin V
PS	Anionic amino-PL	Major PL (15%), located on inner side	12	100
PA	Anionic PL	Minor PL (<1%)	2	100
PG	Anionic PL	Minor PL (<1%)	100	250
PI	Anionic PL	Major PL (7%), mainly located on the inner side	100	50
CL	Anionic PL	Absent from the plasma membrane	15	130
PE	Neutral, amino-PL	Major PL (22%), mainly located on inner side	>8000	100
SM	Neutral, choline-PL	Major PL (9%), located on the outer side	>8000	>8000
PC	Neutral, choline-PL	Major PL (46%), located on the outer side	>8000	>8000

[a] Percentage of total phospholipids, taken from Fridrikkson, *et al.* (54). Percentages may vary for different cell types.

Table 2 *Lack of cross-blocking of binding of 9D2 antibody and annexin V to PS*

PS-binding protein	Competitor[a]	Binding (% control)[b]
Biotinylated annexin V	annexinV	8%
Biotinylated 9D2	annexinV	93%
Biotinylated annexin V	9D2	95%
Biotinylated 9D2	9D2	5%

[a] Annexin V or 9D2 antibody were premixed in 10-fold molar excess over the biotinylated reagents. Binding of biotinylated reagents to PS on microtiter plates was detected by streptavidin-HRP.

[b] Reactivity of biotinylated reagents in the absence of a competitor was taken as 100%. The mean values of triplicate determinations are presented. SD was <10% of the mean value.

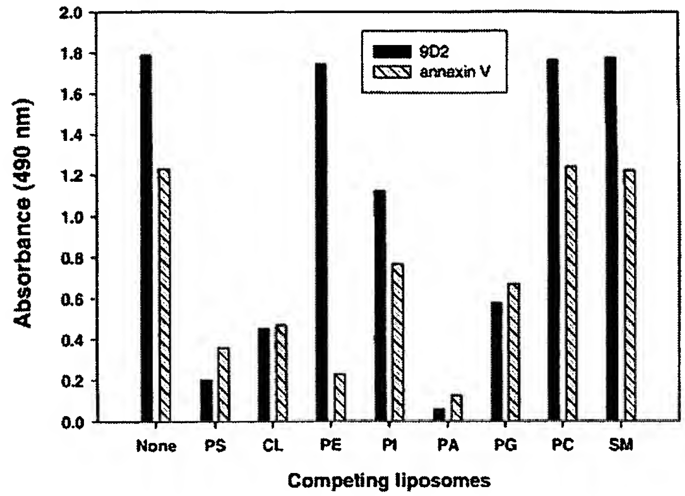

Fig. 2. Competition for binding of 9D2 antibody and annexin V to anionic phospholipids on H_2O_2-treated endothelial cells by liposomes. 9D2 antibody and annexin V (6.7 nM) were preincubated with various phospholipid liposomes (200 μg/ml) in DPBS buffer containing 10% serum. The bound 9D2 antibody and annexin V were detected using goat antirat IgM-HRP and rabbit antiannexin V IgG followed by antirabbit-HRP, respectively. Binding in the presence or absence of competing liposomes was determined as described in "Materials and Methods." SDs of triplicate measurements were <10% of the mean values.

9D2 Antibody and Annexin V Recognize Externalized Anionic Phospholipids on Cell Surfaces. The binding of 9D2 antibody and annexin V to cell surfaces was examined using mouse bEnd.3 endothelioma cells or bovine ABAE cells. Neither 9D2 nor annexin V bound to nonpermeabilized monolayers of either cell type under quiescent conditions. This indicates that the majority of anionic phospholipids of the plasma membrane are normally sequestered to the cytosolic domain. In contrast, strong staining was observed when cells were preincubated with TNF-α and actinomycin D under conditions that caused apoptosis in 90–100% of the endothelial cells.

To confirm that 9D2 and annexin V were binding to phospholipids on cell surfaces, H_2O_2-treated bEnd.3 cells were incubated with 9D2 antibody or annexin V in the presence or absence of various competing liposomes. We had determined previously that anionic phospholipids become exposed on nonapoptotic, viable bEnd.3 cells when they are pretreated with a subtoxic concentration (100–200 μM) of H_2O_2 (43). The binding of 9D2 antibody to H_2O_2-treated bEnd.3 cells was inhibited by liposomes containing anionic phospholipids but not by liposomes containing neutral phospholipids (Fig. 2). The magnitude of inhibition of 9D2 binding to cells varied in the order PA>PS>CL>PG>PI, in close agreement with the results obtained using plastic-immobilized phospholipids (Fig. 1). Similarly, the binding of annexin V to H_2O_2-treated cells was blocked by liposomes containing PS, PA, PE, CL, and, to a lesser extent, PI and PG. Liposomes containing SM or PC did not block annexin V binding to cells, all in agreement with the results obtained using plastic-immobilized phospholipids. These results confirm that 9D2 binds to anionic

Table 3 *Specific localization of 9D2 antibody and annexin V to tumor vessels in mice as judged by indirect immunohistochemistry*

Tissue	9D2 antibody[a]	Rat IgM control	Annexin V[b]
Tumors			
MDA-MB-231	40.6 ± 5.4	0	45.3 ± 5.6
L540cy	19.3 ± 3.3	0	16.7 ± 3.9
NCI-H358	15.6 ± 4.1	0	ND[c]
B16	23.4 ± 4.5	0	21.3 ± 6.6
Meth A	25.7 ± 6.8	0	ND
Normal			
Adrenal	0	0	0
Brain	0	0	0
Heart	0	0	0
Kidney	0[d]	0[d]	0
Intestine	0	0	0
Liver	0	0	0
Lung	0	0	0
Pancreas	0	0	0
Spleen	0	0	0
Testis	0	0	0

[a] Localization of 9D2 antibody and rat IgM control in tumor-bearing mice was determined by injecting the antibody (50 μg) i.v., perfusing the blood circulation of the mice with saline and detecting the antibody on sections of the tissues by using an anti-rat IgM-HRP. The results are presented as the mean (±SE) percentage of PS-positive vessels of MECA 32-stained vessels per field of 0.317 mm^2. Six samples of each type were analyzed. The mean number of MECA 32-positive vessels per 0.317 mm^2 field was 23, 25, 21, 18, and 19 ± 10 vessels for MDA-MB-231, L540cy, H358, B16, and Meth A tumors, respectively.

[b] Localization of annexin V was determined by injecting biotinylated annexin V followed by detection on frozen sections using streptavidin-peroxidase.

[c] ND, not determined.

[d] Nonantigen-specific tubular staining was visible in both 9D2 and control antibody recipients.

phospholipids in the H_2O_2-treated endothelial cells, whereas annexin V binds to PE in addition to anionic phospholipids.

Localization of 9D2 Antibody and Annexin V to Tumor Vessels in Mice. The ability of 9D2 antibody and annexin V to localize to tumor vessels in mice was first determined by indirect immunohistochemistry. Mice bearing various types of solid tumors were injected i.v. with 9D2 antibody or biotinylated annexin V, and 1 h later, were exsanguinated, and the tumors and normal tissues were removed. The tumors were: human MDA-MB-231 breast tumor growing orthotopically in the mammary fat pads of SCID mice; human L540 Hodgkin's tumor growing s.c.; human NCI-H358 NSCLC growing s.c.; mouse B16 melanoma growing s.c., and mouse Meth A fibrosarcoma growing s.c. Frozen sections of tissues were cut and stained with HRP-labeled antirat IgM or with HRP-labeled streptavidin to determine to which cells the 9D2 and annexin V had bound after injection. Blood vessels were identified morphologically and from their positive staining by the pan-endothelial cell antibody, MECA 32, on serial sections.

9D2 antibody and annexin V localized to tumor vessels in all of five tumors included in this study (Table 3; Fig. 3). 9D2 antibody and annexin V gave essentially the same patterns of staining. Vascular endothelium in the tumors showed distinct membrane staining (Fig. 4). The percentage of 9D2 and annexin V-positive vessels ranged from 40% in MDA-MB-231 tumors to 15% in NCI-H358 tumors relative to the number of MECA 32-positive vessels (Table 3). Anionic phospholipid-positive vessels were present on the luminal surface of capillaries and vessels in all regions of the tumors, but were particularly prevalent in and around regions of necrosis. Most anionic phospholipid-positive vessels did not show morphological abnormalities that were apparent by light microscopy. Occasional vessels, particularly those located in necrotic areas, showed morphological signs of deterioration. Localization of the 9D2 antibody to tumor vessels appeared to be specific because membrane staining of tumor endothelium was not observed in tumors from mice that had been injected with rat IgM of irrelevant specificity. Presumably, leakage of the control rat IgM out of tumor vessels occurred to some extent, but the staining of extravascular IgM was too diffuse or too weak to discern by indirect immunohistochemistry. 9D2 antibody and annexin V also localized to the membrane and cytosol of necrotic and apoptotic tumor cells, whereas localization of the control IgM was not detectable (Fig. 3).

No vascular localization of 9D2 antibody or annexin V was observed in 9 of the 10 normal organs that were examined (Table 3). In the kidney, staining of tubules was observed that appeared not to be

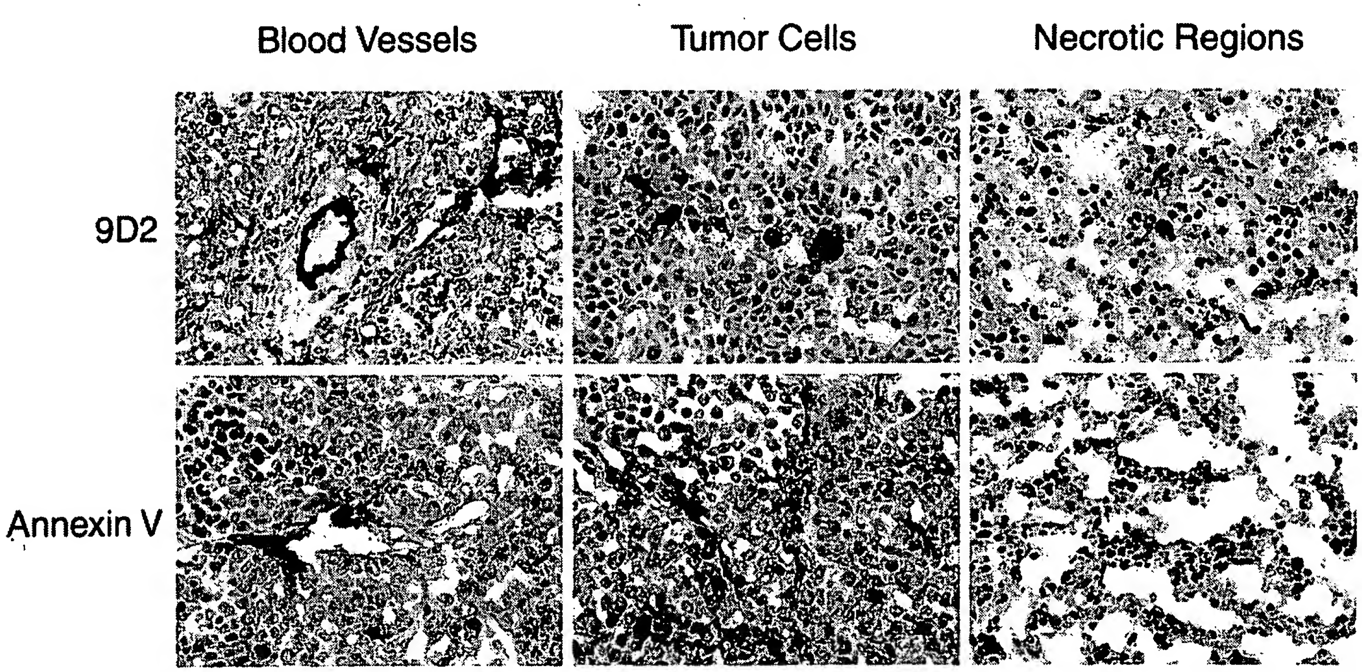

Fig. 3. Localization of biotinylated 9D2 antibody and annexin V to vascular endothelial cells and tumor cells in orthotopic MDA-MB-231 human breast tumors in mice. *Nu/nu* mice bearing MDA-MB-231 tumors in their mammary fat pads were injected i.v. with 50 μg of biotinylated 9D2 antibody or 100 μg of biotinylated annexin V. One h later, their blood circulation was perfused with saline. Tumor and organs were removed and snap-frozen. Localized 9D2 and annexin V were detected on the frozen sections using streptavidin-HRP conjugate. Tumor sections derived from mice injected with saline or control rat IgM served as negative controls.

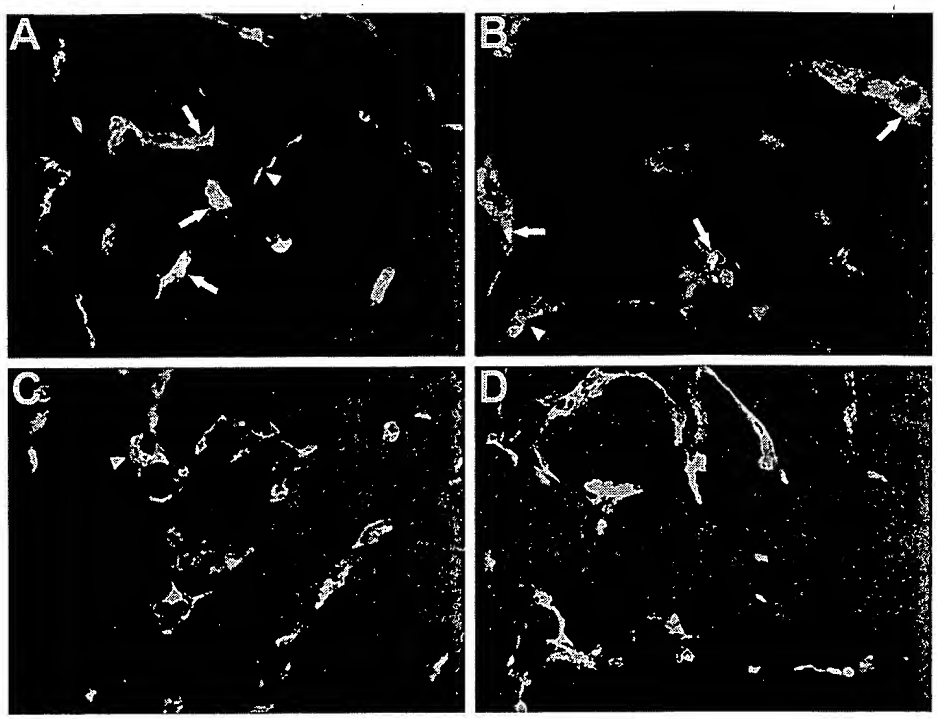

Fig. 4. Colocalization of 9D2 and annexin V labeling with MECA 32 binding. Tumor sections were prepared as described in the legend for Fig. 3. Biotinylated 9D2 antibody (*A*) or biotinylated annexin V (*B*) were detected with a streptavidin-Cy3 conjugate. Sections were then stained with MECA 32 antibody followed by antirat IgG secondary conjugated to FITC. Coincident expression of MECA 32 antigen and localized 9D2 antibody or annexin V is shown by the production of the yellow color on superimposed images (*white arrows*). *White arrowheads* point to MECA 32-positive vessels that did not bind 9D2 antibody or annexin V. Rat IgM of irrelevant specificity did not localize to tumor endothelium. However, leakage of a rat IgM was detected around occasional vessels. Leakage was apparent when the red color extended beyond the green color of the endothelial marker (*C, yellow arrowhead*). Streptavidin-Cy3 did not react with tumor sections derived from noninjected mice (*D*).

antigen specific. Tubules were stained in both 9D2 and control rat IgM recipients, presumably because of secretion of IgM or its metabolites through this organ. The ovaries, a site of physiological angiogenesis, were not examined.

Double-staining experiments were also performed in which mice bearing orthotopic MDA-MB-231 breast tumors were injected i.v. with biotinylated 9D2 antibody, biotinylated control IgM, or biotinylated annexin V. One h later, the mice were exsanguinated, their tumors were removed, and frozen sections were cut. The tumor sections were then stained with Cy3-conjugated streptavidin to detect the biotinylated proteins and with FITC-conjugated MECA32 to detect vascular endothelium. This detection method labeled the biotinylated proteins and the vascular endothelium by red and green, respectively. Where the biotinylated proteins are bound to the endothelium, the converged image appears yellow. The biotinylated 9D2 and annexin V appeared mostly to be bound to the vascular endothelium, because their staining patterns converged with that of MECA 32 (Fig. 4, *A* and *B*). About 40% of MECA 32-positive vessels bound 9D2 and annexin V, in close agreement with the results obtained by indirect immunohistochemistry. However, leakage of the biotinylated proteins into the tumor interstitium was apparent by double staining, whereas it was not apparent by indirect immunohistochemistry. Biotinylated proteins were visible outside the vascular endothelium around a minority (~5%) of vessels. In tumors from mice that had been injected with biotinylated rat IgM of irrelevant specificity, the biotinylated IgM had also leaked into the tumor interstitium around a similar percentage (~5%) of vessels, but mostly appeared not to be bound by the vascular endothelium. (Fig. 4*C*). Presumably, the detection of extravasated 9D2 and annexin V by the double-staining technique but not by the indirect immunohistochemistry technique reflects the greater sensitivity of the former technique and the greater precision with which two staining patterns can be compared. Noninjected control tumors were completely unstained by streptavidin-Cy3 (Fig. 4*D*), indicating that red fluorescence corresponds to a localized protein.

Perturbation of Phospholipid Asymmetry on Tumor Vessels Might Be Induced by Oxidative Stress. Mouse bEnd.3 or bovine ABAE cells *in vitro* were treated for 24 h with various concentrations of factors and conditions that are present in the microenvironment of many tumors (44, 45). Externalization of anionic phospholipids was quantified by measuring ^{125}I-annexin V binding. The amount of annexin V binding was compared with that of cells in which apoptosis of 90–100% of cells had been induced by combined treatment with actinomycin D and TNF-α. Actinomycin D and TNF-α induced the binding of 6.2 pmols of annexin V per 10^6 cells (3.8×10^6 molecules of annexin V per cell) on both cell types, in good agreement with literature reports (46). This value was taken as the maximal level of externalized anionic phospholipids.

Untreated cells were largely devoid of externalized PS, as judged by annexin V or 9D2 binding (Table 4). The basal binding in the presence of growth medium alone was 0.44 and 0.68 pmols of ^{125}I-annexin V for ABAE and bEnd.3 cells, respectively. This corresponds to 7.06% and 10.9% of the maximal binding for ABAE and bEnd.3 cells, respectively, which correlated well with the finding that ~10% of cells bound biotinylated annexin V under the same conditions.

VEGF, hepatocyte growth factor, fibroblast growth factor, TGF-β_1, platelet-derived growth factor, IL-6, IL-8, and IL-10 did not increase binding of ^{125}I-annexin V above the basal level for untreated cells. Inflammatory mediators (IL-1α, IL-1β, TNF-α, and IFN) caused a small but reproducible increase in anionic phospholipid translocation that ranged from 5% to 8% of the maximal level for ABAE cells and from 3% to 14% for bEnd.3 cells (Table 4). Hypoxia/reoxygenation, thrombin, or acidic external conditions (pH 6.8–6.6) induced a moderately high externalization of anionic phospholipid that ranged from 8% to 20% of the maximal level for ABAE cells and from 17% to 22% of the maximal level for bend.3 cells. The largest increase in anionic phospholipid translocation was observed after treatment with 100–200 μM of hydrogen peroxide. This treatment caused nearly com-

Table 4 *Effect of cytokines, growth factors, and stress conditions on exposure of PS on endothelial cells in vitro*

		^{125}I-Annexin V (% of max binding)[b]	
Treatment	Concentration[a]	ABAE cells	bEnd.3 cells
Medium with 10% serum	N/A[c]	0	0
Actinomycin D + TNF-α	50 ng/ml each	100	100
VEGF	20 ng/ml	0	0
TGF-2	20 ng/ml	0	0
Scatter factor	40 ng/ml	0	0
FGF-β_1	20 ng/ml	0	0
PDGF-BB	20 ng/ml	0	0
IL-10	20 ng/ml	0	0
IL-8	20 ng/ml	0	0
IL-6	20 ng/ml	0	0
IL-1α	10 ng/ml	6.4	7.5
IL-1β	10 ng/ml	5.8	5.5
IFN	40 ng/ml	8.6	2.8
TNF-α	20 ng/ml	7.4	13.7
Thrombin	50 nM	8.8	17.4
Hypoxia	1% O_2	17.5	22.5
pH 6.6[d]	N/A	20.2	18.9
Hydrogen peroxide	200 μM[e]	95.5	98.4

[a] Concentrations of cytokines, growth factors, and thrombin were selected from literature values to have maximal stimulatory effect on cultured endothelial cells. These concentrations did not cause toxicity over the period of the test (24 h) as judged by morphological appearance, a lack of detachment, and a lack of uptake of trypan blue.

[b] Binding of ^{125}I-annexin V was performed as described under "Materials and Methods." Basal binding was determined in the presence of growth medium alone. Maximal PS exposure was determined after induction of apoptosis by the combined treatment with actinomycin D and TNF-α. Untreated ABAE and bEnd.3 cells bound 0.44 and 0.68 pmoles of ^{125}I-annexin V, respectively. Maximal binding was 6.2 pmoles of ^{125}I-annexin V for both cell types (equivalent to 3.8×10^6 molecules per cell). The percentage of increase of annexin V binding was calculated according to the following formula: (net experimental binding/net maximal binding) $\times$ 100. Average of duplicates from three separate experiments is presented. SD was <10%.

[c] N/A, not applicable.

[d] Cells were exposed to the growth medium lacking bicarbonate that had been adjusted to pH 6.6 with 1N HCl. Cells were incubated at 37°C in the absence of CO_2.

[e] The maximal concentration of H_2O_2 that did not cause cytotoxicity under chosen conditions.

plete (95%) externalization of anionic phospholipid in both cell types as judged by ^{125}I-annexin V binding (Table 4). More than 70% of ABAE and bEnd.3 cells bound biotinylated annexin V, as judged immunohistochemically (data not shown). Endothelial cells in which anionic phospholipid translocation was generated by treatment with hypoxia/reoxygenation, thrombin, acidity, TNF-α, IL-1, or H_2O_2 remained attached to the matrix during time period of the assay (24 h), retained cell-cell contact, and retained their ability to exclude trypan blue dye. Normal anionic phospholipid orientation was restored 24–48 h later in the majority of the cells after the inducing factor was removed or the culture conditions were returned to normal. These results indicate that hypoxia/reoxygenation, acidity, thrombin, and inflammatory cytokines all trigger a transient translocation of anionic phospholipid on viable endothelial cells.

Combined Effects of Inflammatory Cytokines and Hypoxia/Reoxygenation on Anionic Phospholipid Exposure by Endothelial Cells *in Vitro*. Enhanced anionic phospholipid exposure was observed when ABAE cells were subjected to hypoxia/reoxygenation in the presence of IL-1α or TNF-α. In the absence of the cytokines, hypoxia/reoxygenation conditions increased PS exposure to 15% of the maximum level for cells treated with apoptotic concentrations of actinomycin D and TNF-α. In the presence of subtoxic concentrations of IL-1α or TNF-α, hypoxia/reoxygenation increased anionic phospholipid exposure to 26% and 33%, respectively, of the maximum (Fig. 5). Cytokines in the absence of hypoxia/reoxygenation increased annexin V-binding sites by <7% indicating that the combination of cytokines and hypoxia/reoxygenation had greater than additive effects on PS-exposure. Thus, in tumors, the exposure of anionic phospholipids induced by hypoxia/reoxygenation may be amplified by inflammatory cytokines and possibly by such other stimuli as acidity and thrombin.

DISCUSSION

The major finding to emerge from this study is that anionic phospholipids are exposed on the surface of tumor endothelium. This phenomenon was demonstrated using two independent reagents that bind selectively to anionic phospholipids: a monoclonal antibody, 9D2, and annexin V.

9D2 antibody and annexin V bound with high affinity and specificity to anionic phospholipids adsorbed to plastic, as liposomes, or presented on the membrane surface of activated or apoptotic endothelial cells *in vitro*. 9D2 bound strongly to PS, PA, and CL but more weakly to PI and PG. Annexin V bound to PE in addition to PS, CL, PA, PI, and PG, as found previously by others (40–42, 47). Recognition of anionic phospholipids by 9D2 antibody was identical in the presence and absence of serum, indicating that binding does not require serum cofactors. Binding of 9D2 to anionic phospholipids did not require Ca^{2+} ions, whereas the binding of annexin V required Ca^{2+}. Cross-blocking experiments on PS-coated plates showed that 9D2 and annexin V do not block each other's binding to PS. This indicates that the two reagents recognize different epitopes on the PS molecule, or, more likely, differently packed forms of PS. Annexin V is thought to bind to planar PS surfaces, whereas anti-PS antibodies are thought to bind to hexagonally packed PS (48). Both forms are probably present on PS-coated plates.

9D2 antibody and annexin V specifically localized to tumor vessels, and to tumor cells in and around necrotic regions of tumors, after i.v. injection into tumor-bearing mice (Fig. 3). Between 15% and 40% of blood vessels (Table 3) in all five types of tumors that we examined *in vivo* had anionic phospholipid-positive endothelium. In contrast, none of the blood vessels in normal tissues had detectable externalized anionic phospholipids. The specificity of staining of tumor endothelium by 9D2 was demonstrated by: (*a*) coincidence of staining by 9D2 and the pan-endothelial antibody, MECA 32, as detected by indirect immunohistochemistry and double-staining techniques (Figs. 3 and 4); (*b*) the weaker and more infrequent staining of vascular endothelium in tumors by control rat IgM; (*c*) the finding that extraction of phospholipids from tumor sections with detergents or organic solvents

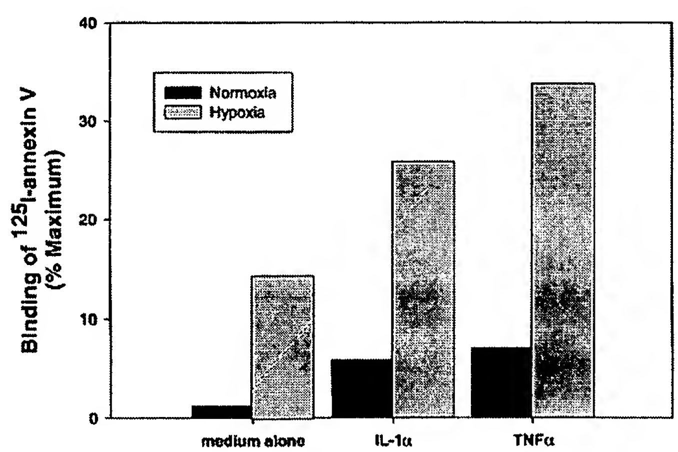

Fig. 5. Combined effects of hypoxia and inflammatory cytokines on PS exposure. bEnd.3 cells were treated for 24 h with IL-1α and TNF-α under normoxic (*black bars*) and hypoxia (*gray bars*) conditions. The cell monolayers remained intact and able to exclude trypan blue dye under these conditions. PS externalization was determined by measuring binding of ^{125}I-annexin V. The level of PS exposure was expressed as a percentage of that on cells treated with a combination of actinomycin D and TNF-α as explained in "Materials and Methods."

abolished staining; (*d*) the lack of localization of 9D2 or annexin V to the quiescent endothelium in normal organs; and (*e*) the blocking of 9D2 or annexin V binding to H_2O_2-treated endothelial cells *in vitro* by liposomes prepared from anionic phospholipids but not neutral phospholipids.

The main anionic phospholipid that is localized by 9D2 or annexin V on tumor vasculature is likely to be PS. This is the most abundant anionic phospholipid, and its exposure on the cell surface is known to be regulated by environmental influences or injury. We cannot exclude the possibility that other anionic phospholipids (*e.g.*, PI, PA, and PG) are also exposed, but they are less abundant and their membrane position is not as tightly regulated by environmental conditions. However, it is possible that the major neutral phospholipid, PE, is also exposed on tumor endothelium and contributes, together with PS, to the annexin localization that we observe on tumor vessels. The position of PE in the plasma membrane is regulated in a similar manner to PS. PE is segregated to the internal leaflet of the plasma membrane in part by aminophospholipid translocase, although at a slower rate than PS (49), and is transported to the external surface by scramblase (50). Recent work has shown that PE, like PS, is exposed during apoptosis and cell activation (51).

To examine the mechanism of exposure of anionic phospholipids on tumor endothelial cells, a series of experiments was performed in which endothelial cells *in vitro* were treated with various factors and conditions known to be present in the tumor microenvironment. Hypoxia followed by reoxygenation, acidity, and thrombin increased PS exposure on viable endothelial cells to between 10% and 22% of the level seen when all of the cells are apoptotic (Table 4). Inflammatory cytokines (TNF-α and IL-1) also caused a weak but definite induction of PS exposure. Our findings (Fig. 5) are consistent with the possibility that, in tumors, exposure of anionic phospholipids on the vascular endothelium is induced by hypoxia/reoxygenation in combination with inflammatory cytokines, thrombin, and acidity. ROS may be generated by tumor cells as a byproduct of metabolism or in response to hypoxia (30). Cytokines released by tumor cells may induce leukocyte adhesion molecules on the endothelium that mediate adherence of activated macrophages, polymorphonuclear cells, and platelets to tumor endothelium and additional secretion of ROS. The ROS may then induce PS translocation through oxidation of thiol-containing transport molecules or peroxidation of lipids (52), possibly by causing an influx of Ca^{2+} or release of Ca^{2+} from intracellular stores (53). It is also possible that exposure of anionic phospholipids occurs during certain stages of cell division and contributes to the staining of the proliferating endothelium in tumors: the relationship between angiogenesis and exposure of anionic phospholipids was not examined in the present study.

Exposure of PS and other anionic phospholipids may in part explain the procoagulant status of tumor endothelium that has long been recognized (26). The anionic phospholipids would provide the surface on which coagulation factors concentrate and assemble (24, 25). It also would provide an attachment site for circulating macrophages (21), T lymphocytes (17), and polymorphonuclear cells that assists in leukocyte infiltration into tumors.

Antibodies, annexins, and other ligands that bind to anionic phospholipids might be used for the targeting or imaging of tumor blood vessels. Anionic phospholipids are attractive as tumor vessel targets for several reasons: they are abundant (PS is present at $>10^6$ molecules per cell); they are on the luminal surface of tumor endothelium, which is directly accessible for binding by vascular targeting agents in the blood; they are present on a significant percentage of tumor endothelial cells in diverse solid tumors; and they appear to be absent from endothelium in all of the normal tissues. However, PS may be exposed on vascular endothelium in nonmalignant lesions (*e.g.*, sites of inflammation), where cytokines, hypoxia, and ROS might induce PS translocation. It is possible this could lead to toxicity with a vascular targeting strategy, making it necessary to exclude patients with these conditions from treatment. PS is also present on cells undergoing physiological apoptosis, but, because these cells are destined to die, toxicity through targeting these cells is of lesser concern. PS is also found on the surface of apoptotic vesicles, such as are shed from tumors. These could bind to anti-PS antibodies in the blood circulation or diminish localization to tumor vasculature; nevertheless, access of the antibody to tumor endothelium does not appear to be a problem in the present studies performed in mice.

In conclusion, anionic phospholipids on tumor vessels may provide target molecules for tumor therapy. In addition, anionic phospholipids exposed on apoptotic and living tumor cells may contribute to imaging intensity. Annexin V and antibodies to anionic phospholipids might be used to deliver a cytotoxic drug, radionuclide, or coagulant to tumor vessels for the vascular targeting of tumor vessels in humans.

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